AGONIST-ANTAGONIST INTERACTIONS IN THE CENTRAL NERVOUS SYSTEM

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Due to the complex nature of the neuropharmacological experiments described in this thesis, many of the experiments were conducted in collaboration with Professor D.R. Curtis. Dr J. Davies (The School of Pharmacy, London) participated in some of the experiments described in Section V and in all of the experiments in Section VI. Dr C.J.A. Game participated in the experiments described in Sections III, IV, V, VII and VIII.

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1. GENERAL INTRODUCTION

The aim of this work was to investigate the following aspects of the interaction of agonists and antagonists in amino acid-mediated transmission in the feline central nervous system:

(1) The 'active' conformations of GABA and L-glutamic acid for interaction with their respective post-synaptic receptors, conformations suggested by a study of the structure-activity relationships of analogues of GABA and L-glutamic acid having restricted conformations.

(2) The differentiation of two distinct populations of excitatory amino acid postsynaptic receptors (L-glutamate and L-aspartate populations) by the use of relatively selective agonists for each type of receptor population.

(3) The mechanism of action of certain neurotoxins and convulsants and the structure-activity relationships of these substances.

(4) The identification of new, selective antagonists of the postsynaptic actions of putative inhibitory or excitatory amino acid transmitters.

The general introduction to this thesis thus reviews the present state of knowledge concerning the 'active' conformations of GABA and L-glutamate, and discusses the use of analogues of restricted conformation to analyse GABA, L-glutamic and L-aspartic acid receptors. Micro-electrophoretic methods have been used extensively in the

experiments described in this thesis and in the quoted literature. The data obtained from this type of investigation must be interpreted with considerable caution because of the complexities of the method and the necessary assumptions upon which interpretations are based. For this reason the technique has been reviewed in parallel with the discussion of structure-activity relationships revealed in this and related investigations.

The chemical nature of synaptic transmission in the mammalian central nervous system is now well accepted, and the interactions of transmitters, putative transmitters, related agonists and antagonists with their appropriate receptors has formed the basis of considerable pharmacological investigation. The purpose of this introduction is to review certain aspects of the microelectrophoretic use of agonists and antagonists in structure-activity relationship (SAR) studies, and in studies designed to identify the transmitters of particular pathways in the mammalian central nervous system (CNS) which are believed, on the basis of pharmacological and neurochemical evidence, to be mediated by simple amino acids (see Curtis and Johnston, 1974a).

Although autoradiographic techniques have become available for identifying and quantitating receptor areas on excitable membranes, for example, the mouse neuromuscular junction (Barnard, 1973; Barnard, Wieckowski and Chiu, 1971), and improved techniques have been reported for the isolation of a 'receptor' protein, the nicotinic cholinoceptive

receptor protein (Heilbronn and Mattson, 1974), these techniques are limited and it is still not possible to study transmitter-receptor interaction at the atomic or molecular level. This problem continues to be attacked indirectly by studying the structure and activity of selected compounds, which act on a common receptor, to either mimic or antagonise the action of putative transmitters. The basic assumption in the general analysis of SARs is that the agonist or antagonist has a structure which, to some extent, is complementary to its receptor site on the membrane, and that progressive changes in the structure of the agonist or antagonist molecule can be correlated with changes in its pharmacological activity. The validity of this general analysis is challenged by at least three major problems. First, the neurotransmitters and many of their analogues are small, relatively flexible, molecules and the conformations of these molecules, as receptor-bound species, may bear no relationship to the conformations that can be determined in solution or in the crystalline state. Second, there could be multiple modes of binding between ligand and receptor. Third, the differences in pharmacological activities of a series of analogues may also reflect differences in the processes that link the initial analogue-receptor interaction with the final measured response. The chain of events that occurs after agonist-receptor interaction is only poorly understood, and hence the physiological response that is measured should be as closely related to

the initial interaction as possible. Despite these constraints, which will be considered again when specific examples are discussed, SAR studies have been widely employed in attempts to determine the molecular basis for neurotransmitter action.

The use of agonists to analyse receptors

The first attempts to analyse the structure-activity relationships of pharmacologically active compounds were directed towards drugs active at acetylcholine receptors. Raventós (1937) made the first quantitative study of the relative potency of a series of quaternary ammonium salts in terms of potency in causing contracture of various isolated muscle preparations. Such isolated preparations allow a quantitative treatment of agonist-receptor interactions, based on the assumption that the agonist is uniformly distributed throughout the bathing fluid and tissue. *In vitro* preparations of the mammalian CNS, however, are not available for such studies, and the structure-activity relationship analysis must be conducted *in vivo*. The effects of various substances on the CNS can be observed after administering them directly to the tissue, for example by microinjection, or after indirect administration through the blood supply of the tissue. These methods of administration, however, are often unsatisfactory because of distribution problems and the possible longlasting and wide reaching effects of a systemically administered substance. Microelectrophoresis has been used as a means of overcoming some of these problems. This technique, of using an electrical current

to control the ejection of chemical substances from fine glass micropipettes, was first developed by Nastuk (1953) and del Castillo and Katz (1955) as a means of administering small quantities of acetylcholine to single muscle fibres. Curtis and R.M. Eccles (1958) later developed this technique using multibarrel micropipettes to investigate systematically neurones in the CNS.

Microelectrophoretic considerations

Details of the microelectrophoretic technique have been published by several workers (Curtis, 1964; Krnjević, 1964; Salmoiraghi and Weight, 1967), and the techniques employed by Curtis are outlined in more detail in the Methods section of this thesis (Section II). The main advantage of the microelectrophoretic method is that it allows small, controlled quantities of substances to be delivered directly into the extracellular environment of individual neurones in intact preparations of the CNS. Diffusional barriers, such as the blood brain barriers, are by-passed, and the action of a substance ejected from a micropipette is restricted to a small volume of tissue adjacent to the tip of the micropipette. Neuronal activity can be recorded by means of a microelectrode adjacent to the micropipette used to administer substances, and observed alterations in neuronal activity cannot arise indirectly from an action upon peripheral receptors or other distant neurones having synaptic connections with the neurone under observation. The main disadvantages of the technique are those which arise from the use of an

electric current to eject substances which are ionised to varying degrees from what may be considered as point sources within a non-uniform medium. Changes in neuronal activity produced by current flow per se can usually be distinguished from direct drug effects (see Curtis, 1964), but the evaluation of the concentration of ejected agents, a parameter of major importance in determining the relative potency of agonists, presents certain difficulties. Since the electrical resistance of the drug-containing micropipette is high, compared with that of the external medium which lies between the pipette orifice and an indifferent electrode, the potential across the medium associated with the electrophoretic ejecting current is small (Curtis, Perrin and Watkins, 1960). Thus the movement of ejected substances within the medium is not significantly controlled by potential gradients across it, and the concentrations which are attained in the environment of the tip of the micropipette depend upon the rate of ejection of the agent and the nature of the external medium.

The electrophoretic ejection of substances from solutions contained within glass micropipettes involves both iontophoresis and electro-osmosis. The rate of ejection of a fully ionised substance, by iontophoresis alone, is directly proportional to the current and transport number of the particular ion (i.e. the fraction of the current carried by that ion). It is not possible, however, to determine the actual rate of iontophoretic ejection by measuring the current, for the transport number depends upon the mobility of the ion and such factors as

the total ionic concentration, especially within and just outside the tip of the micropipette, and these concentrations can only be known approximately for an *in vivo* preparation. The determination of the total electrophoretic flux from the measured electrophoretic current is further complicated by the contribution that electroosmosis makes to the total flux. Attempts have been made to calibrate the rate of ejection of compounds for which there are suitable chemical, physical or biological assay methods (see Curtis, 1964; Krnjević, Laverty and Sharman, 1963; Krnjević, Mitchel and Szerb, 1963). The *in vitro* conditions that were employed for these calibrations (especially the current and time of ejection) were not similar, however, to the *in vivo* experimental conditions. Radioisotopic techniques have been recently applied to this problem, and it has been possible to measure rates of ejection with currents and ejection periods that were similar to those used for the microelectrophoretic testing of the compounds that were radioisotopically estimated. Among the substances ejected, and radiosiotopically estimated *in vitro*, the microelectrophoretic release of glutamate (Zieglgänsberger, Herz and Teschemacher, 1969; Obata, Takeda and Shinozaki, 1970; Gent, Morgan and Wolstencroft, 1974), aspartate (Gent, Morgan and Wolstencroft, 1974), GABA (Obata, Takeda and Shinozaki, 1970; Zieglgänsberger, Sothmann and Herz, 1974), glycine (Zieglgänsberger, Sothmann and Herz, 1974), and noradrenaline (Bradley and Candy, 1970; Hoffer, Neff and Siggins, 1971; Bradshaw, Roberts and Szabadi, 1973; Candy, Boakes, Key and Worton, 1974) has been shown to be directly

proportional to the ejecting current. The transport numbers that were calculated from these determinations, however, indicated that there was considerable variability between pipettes of the same size when tested under the same conditions. Thus the rate of ejection of any one substance may vary between different micropipettes when the same currents are passed.

Even if it is possible to estimate the amount of a substance that is released from a micropipette by an electrophoretic current, the concentration developed in the region of a particular neurone is still exceedingly difficult to predict, for the second factor upon which the concentration depends, the nature of the external medium is a very complex one. If the orifice of the micropipette is regarded as a continuous point source within a uniform medium, then the concentration at any particular distance from the source can be estimated using the principles of diffusion (for this treatment see Carslaw and Jaeger, 1959; Curtis, Perrin and Watkins, 1960; Jaeger, 1965), provided the rate of ejection and the diffusion coefficients are known. Because of the difficulty in measuring the distance from the orifice to the point at which it is desired to know the concentration, an alternative approach has been to consider the micropipette orifice as an instantaneous point source in a uniform medium. Using this approach the theoretical concentration resulting from a brief electrical pulse has a peak value which can be calculated from the observed time to peak response, the

quantity of drug ejected and the diffusion coefficient, without estimating the distance from orifice to cell membrane (see del Castillo and Katz, 1955; Ogston, 1955). These calculations, however, are of limited practical value due to the complex anatomical arrangement of the extraneuronal space within the CNS.

The 'extraneuronal' space into which microelectrophoretic ejection occurs is presumably a space which is larger than the extra cellular space that is seen in electronmicrographs, and it is partly created by the relatively large tip of the micropipette. The path between the tip of the micropipette and the true extraneuronal space is probably barred by tissue debris, glial cells and any diffusional barriers that may be intimately associated with the synaptic areas on neurones (Eccles, J.C., Eccles, R.M. and Fatt, 1956). It is unlikely that uniform diffusion occurs in such an environment, and hence it is unlikely that the receptors located upon any one neurone are subjected to a uniform concentration of an electrophretically ejected agent, unless the distance over which the receptors are distributed is small compared with the diffusion path. After electrophoretic ejection into the extracellular environment an ejected agent not only passively diffuses, but it may also interact physically or chemically with one or more components of the extracellular medium. It is conceivable that an ejected substance could be rendered pharmacologically inert by the formation of complexes with components

of the extracellular medium, but the possible active uptake and enzymic modification of ejected agents that are similar to the neurotransmitters (Curtis, Duggan and Johnston, 1970), is even more likely to modify the concentration of an ejected agent from the theoretically predicted concentration.

It is thus virtually impossible to determine the active concentration, at the receptor site, of an agent that has been ejected electrophoretically. Despite this problem assessment of the relative potencies of a series of agonists can be made by using multibarrel micropipettes such that the orifices are located at approximately the same distance from a given neurone. Under the usual recording conditions in the CNS, the diffusion paths can be assumed to be very similar, provided the compounds to be compared act upon the same receptors. Such tests would then, presumably, be subjected to the same uncontrolled variables, provided the compounds were similar in behaviour regarding diffusion, uptake, binding etc. The only remaining systematic error arises from the assumption that equal rates of administration are produced by equal electrophoretic currents. Even using the same micropipette the measured relative potency of a group of agonists can vary by as much as 30% (Curtis and Watkins, 1963) when estimates are obtained on several neurones. This difference probably reflects variation in the relative spatial disposition of the neurones and the micropipette orifice, variation in extracellular environment and variation in rates of ejection. It has been reported that rates of

ejection of isotopically labelled noradrenaline into saline or Ringer's solution can be either similar to (Candy, Boakes, Key and Worton, 1974) or differ from (Hoffer, Neff and Siggins, 1971) that into brain slices, whilst the ejection of tritium-labelled acetylcholine *in vitro* agreed with the *in vivo* ejection rate (Bradley and Candy, 1970). Furthermore, in view of the variable amount of tissue debris that can 'cap' microelectrodes during penetration of central nervous tissue (Andersen and Curtis, 1964; Yamamoto, 1967), it is not surprising that the rates of ejection of isotopically labelled L-aspartate and L-glutamate, as measured *in vitro* into saline, before and after penetration of the feline brain stem, differed (Gent, Morgan and Wolstencroft, 1974). Hence it is not possible to make accurate, direct comparisons of potencies in terms of electrophoretic currents, unless the rates of ejection during the test can be measured. In practice, it is not always possible to obtain such measurements due to the unavailability of radioactively labelled compounds, or because the nature of the experiment does not allow tissue sampling after each estimation of potency. The *in vivo* measurement of rates of ejection are also questionable because the distribution volume of the ejected compound, or its labelled metabolites, may exceed the volume of tissue that is sampled for measurement. At present, there appears to be no way in which the problem of accurately determining the rate of electrophoretic ejection *in vivo*, and its relationship to extraneuronal concentration, can be overcome to render the technique

more quantitatively reliable. By performing a number of experiments with many cells, and by using different micropipettes in which the combination of test compounds is varied, an assessment can be made of the relative potencies of a series of substances assuming that the rates of ejection, and therefore the local tissue concentrations, are directly proportional to the electrophoretic currents which are used. It must be remembered, however, that any differences in potency that are so demonstrated may be partially explained by the factors discussed above, viz:

(i) Apparent transport numbers may vary from compound to compound, from micropipette to micropipette and may not be constant for any one micropipette.

(ii) The 'effective diffusion coefficients' of different substances in central nervous tissue may vary, similarly rates of inactivation or uptake by the tissue may vary and hence the volumes of tissue affected by ejected substances may differ. Consequently, for equal ejection rates different numbers of receptors could be affected, and by different concentrations of substances.

(iii) Substances may be acting on different receptors, which are present in their respective tissue distribution volumes in different numbers. Alternatively, there may be any one of a number of functionally overlapping receptor populations.

For these reasons no great accuracy can be claimed for this comparative method. Finally, it is not possible to determine whether a substance is more potent in terms of its affinity for a receptor or its intrinsic activity (Ariëns, 1954). Despite these limitations upon the microelectrophoretic method, when used for determining relative potencies, the information so obtained can be of considerable assistance in neuropharmacological studies, and two examples of such studies involving the excitatory and the inhibitory amino acid receptor will now be discussed.

The excitatory amino acid receptor(s)

Considerable neurochemical and neuropharmacological evidence has been collected to suggest that both L-glutamate and L-aspartate could be excitatory transmitters of afferent and efferent pathways within the mammalian CNS (for recent reviews see Curtis and Johnston, 1974a; Krnjević, 1974). Microelectrophoretically administered L-glutamate and structurally related amino acids excite feline central neurones by a reversible depolarisation accompanied by an increase in membrane conductance (Curtis, Phillis and Watkins, 1960; Krnjević and Schwartz, 1967; Ziegelgänsberger and Puil, 1973a). L-Aspartate reversibly depolarises feline motoneurones in a manner similar to that of L-glutamate (Curtis, Phillis and Watkins, 1960). By comparing the potencies of a series of acidic amino acid analogues as excitants of feline neurones (Curtis and

Watkins, 1960; Curtis and Watkins, 1961; Curtis and Watkins, 1963; Crawford and Curtis, 1964; Krnjević and Phillis, 1963) definitive structure-activity relationships concerning these excitant substances have emerged. By extensively modifying the chain length and the charged groups, and by assuming that the analogues acted on a common receptor, a three point receptor was hypothesised to account for the data (Curtis and Watkins, 1960). The most significant features arising from these studies are as follows. Excitatory activity is associated with two acidic (anionic) groups and one basic (cationic) group, the anionic groups being separated by a distance apart which approximates the distance between the α- and ω-carboxylate groups in the glutamate and aspartate molecules. The cationic group is positioned close to the α-carboxylate (anionic) group, and it appears that the optimum position is α with respect to the cationic group. Excitatory activity was abolished if the acidic or basic groups were not free (that is converted to esters or amides), and was reduced or abolished by substitutions within the intermediate carbon chain or upon the amino group, depending upon the size, number and position of the substituents. N-Alkylation of aspartic acid produced a compound, the potency of which depended upon the size of the substituent and whether the parent compound was D- or L-aspartic acid. N-Alkylation of D- or L-glutamic acids produced compounds of potency approximately equal to, or lower than, the parent compound. The conclusions, however, that could be drawn from this data were restricted by the

possible conformations that glutamic and aspartic acids and their analogues can adopt.

The problem of determining the conformation of a receptor-bound ligand has been approached from at least four different directions. X-ray crystallography can be used to elucidate the structure of a ligand in the crystalline state, but the relevance of the application of structural data obtained for the crystal lattice to considerations of the conformation of a receptor-bound ligand can be questioned. This same criticism must arise from the second approach to the problem, the extrapolation of conformational data obtained for a ligand in solution by proton or neutron magnetic resonance methods. Partington and co-workers have used the neutron magnetic resonance spectra of a series of cholinergic agonists, and the Karplus equation, to calculate the fractional population of the three particular conformations, assumed to be of low energy, that would be found in a solution of each of the agonists (Partington, Feeney and Burgen, 1972). What appeared to be a rational use of the neutron magnetic resonance data, however, demonstrated no apparent correlation between the predominant conformation found and the reported activity of the agonist. A third approach to the problem is completely theoretical in that extended Hükel theory (Hoffman, 1963) has been used to determine preferred conformations of agonists. Such calculations treat the molecules as if they were <u>in vacuo</u>, but biological processes do not occur in the conservative state, and hence these calculations would be more relevant if they

could be extended to include interaction between the agonist, water and functional groups of potential relevance to the receptor system. A fourth approach is to consider the activity of structurally rigid, or semirigid analogues of the agonist in which the number of possible conformations is reduced. By studying the SARs of these compounds, common three dimensional features may be more readily demonstrated in the analogues and the parent compound. It is obvious that an analogue of restricted conformation should preferably have a structure that does not deviate too significantly from that of the parent compound, for the molecular changes that produced the structural rigidity could also alter the pharmacological activity of the molecule.

Two conformationally restricted analogues of glutamic acid, ibotenic and kainic acids, have been shown to excite central neurones (Johnston, Curtis, de Groat and Duggan, 1968; Shinozaki and Konishi, 1970), but the SARs of these and other conformationally restricted analogues of glutamic and aspartic acids have not been fully investigated. An examination of the structures of ibotenic acid, kainic acid and other excitant analogues of glutamic acid that are conformationally restricted, may assist in determining whether glutamate interacts with its receptor in a folded or extended conformation. Van Gelder (1971) has suggested that glutamate combines with its receptor in a folded conformation. His argument is based on the suggestion of Curtis and Watkins (Curtis and Watkins, 1965) that L-glutamate and L-aspartate interact with the

same receptor, and consequently there is a high probability that the equivalent ionised groups should adopt sufficiently congruent conformations to combine with an identical receptor. Van Gelder supports his argument with the observation (Curtis and Watkins, 1965) that dicarboxylic acids which have four carbon chains and are otherwise identical to L-glutamate and L-aspartate display no excitant activity, and the distance between terminal cationic (basic) and anionic (acid) groups are in excess of the equivalent distance in the folded glutamate or aspartate molecule. The assumption, however, that L-glutamate and L-aspartate must have the same dimension between equivalent interactive groups for interaction with a common receptor is questionable, for an hypothetic receptor could undergo conformational changes to interact with ligands of slightly different dimensions. Furthermore, L-glutamate and L-aspartate may interact with different receptors, being the excitatory transmitters of different excitatory pathways.

The incorporation of semirigid three dimensional structures into the molecules of L-glutamate and L-aspartate, and subsequent investigations of their pharmacological activities, could assist not only in elucidating their structure-activity relationships, but could also be of assistance in distinguishing aspartate-mediated from glutamate mediated excitatory pathways in the CNS. Apparent differences in the sensitivity of neurones to glutamate and aspartate may indicate two populations of receptors - one for aspartate and one for glutamate -

(which may or may not overlap) and hence a predominance of one type of amino acid releasing terminal on the particular neurones that are being studied. For example, it has been reported that feline Renshaw cells are more sensitive to electrophoretically administered L-aspartate than to L-glutamate, whereas spinal dorsal horn interneurones are more sensitive to L-glutamate than to L-aspartate (Duggan, 1974). Hence there is a differential sensitivity of the two types of neurones and this difference may indicate a greater proportion of glutamate than aspartate receptors on dorsal horn interneurones and the reverse for Renshaw cells. Furthermore, it may be possible to correlate the differential sensitivity of the two types of neurones with a predominance of one type of excitatory synapse on dorsal horn interneurones, and another type on Renshaw cells (see Section VII).

The structural similarity and flexibility of the glutamate and aspartate molecules makes it conceivable that if there is a population of receptors for each acid, then there could be an overlap in the populations of the glutamate and aspartate receptors, in which case it may be difficult to demonstrate significant differences in the sensitivity of specific populations of neurones to these two excitants. The use of conformationally restricted analogues of these two amino acids, which interact more selectively with one of these receptors, could improve the probability of detecting such relative sensitivity differences.

The microelectrophoretic determination of the differential sensitivity of neurones suffers from many of the interpretational problems that have been previously outlined for the determination of differential potency of electrophoretically administered agents. In comparing the differential sensitivity of different types of neurones, however, the problem of different rates of electrophoretic ejection is reduced (see Duggan, 1974). In these types of experiments an approximately equal number of different types of neurones must be tested with any one micropipette, in which case the apparent transport numbers for the substances being ejected are expected to remain relatively constant, and thus should be of minor importance to the determination of relative sensitivity. In practice it is likely that the apparent transport numbers could alter between tests, for example if variable 'capping' of the micropipette was occurring (Andersen and Curtis, 1964), or if ejection of an agonist was accompanied by appreciable electrical noise and changes in electrical resistance (Zieglgänsberger, Herz and Teschemacher, 1969). It is also possible that the histological and neurochemical environments of the two cell types may differ sufficiently to cause either, or both, test substances to be treated differently in the two environments, resulting in different relative diffusion patterns and different relative volumes of tissue being affected by the ejected substances. If the receptors are located only postsynaptically, and if the distribution of the relevant types of synapses can be histologically

or physiologically determined, the difference in observed sensitivity may be explained by a difference in the distribution of the receptors relative to the tissue distribution volumes of the ejected substances. In addition, either test substance could have a different affinity for or different intrinsic activity (Ariëns, 1954) at receptors on different types of neurones, and because of the present inability to determine the concentration of ejected substance at the active site, it is not possible to calculate these parameters. It is also not possible, at present, to determine whether the diffusion, uptake or physical inactivation of ejected substances differs between two types of neuronal environments in the CNS. The final explanation for the observation of different relative sensitivities, that the two cell types have different relative proportions of receptors, can thus only be used to support additional neurochemical or pharmacological evidence that indicates a predominance of one type of synapse on the cells in question.

The inhibitory amino acid receptor

There are now reasonable grounds for considering glycine and gamma-aminobutyric acid (GABA) as transmitters mediating certain inhibitions in the mammalian CNS (see Curtis and Johnston, 1974a; Krnjević, 1974). In this section, however, the emphasis will be placed on GABA and its receptor, an aspect that is further investigated in this thesis. GABA depresses the firing of neurones

throughout the mammalian CNS, and hyperpolarisation of the neuronal membrane with an increase in conductance has been demonstrated in spinal and supraspinal regions of the feline CNS (Curtis and Johnston, 1974a; Krnjević, 1974). From a comparison of the electrophoretic potency of a series of GABA analogues (Crawford and Curtis, 1964; Curtis and Watkins, 1960, 1961, 1965; Krnjević and Phillis, 1963) the following general requirements for depressant activity have been reported. Depressant activity is associated with amino acids containing one acidic (COOH, SOOH or SOOOH) and one basic group (NH_3). An excitatory amino acid can be converted to an approximately equipotent depressant amino acid by α-decarboxylation. The optimum separation of the basic and acidic groups is two or three carbon atoms, if the amino group, however, is substituted by a guanidino group the chain must be somewhat shortened. Substituents on carbon atoms within the chain, or on the nitrogen atom, reduce or abolish depressant activity according to their position, size and number. The acidic and basic groups must be free.

The GABA molecule, like many of its analogues, is flexible and hence, as with glutamate, there is considerable difficulty in determining its conformation when in the receptor-bound state. X-ray diffraction methods have been used to determine the structure of GABA (Tomita, 1971; Steward, Player, Quilliam, Brown, and Pringle, 1971; Warner, Player and Steward, 1973), the results of which reveal a partially folded molecule with nitrogen-oxygen distances of about 4.2 and 5.75 Å. This conformation,

within the crystal state where each molecule is influenced by crystal packing forces, may also be of importance when GABA is in close association with the receptor and again inter-molecular forces are present. Molecular orbital calculations, however, have predicted a fully extended conformation. Kier and Truit (1970) used extended Hückel theory (Hoffman, 1963) in which the conformation associated with the lowest calculated energy is predicted to be the preferred conformation, and the results suggested that the preferred GABA conformation was fully extended with nitrogen to oxygen distances of 5.0 to 6.1 Å. Similar calculations have been performed for the GABA analogue β-hydroxy GABA (Kier and George, 1973) and two equivalent conformational preferences were predicted. The first preference had an oxygen to nitrogen distance that was identical to that found for GABA using molecular orbital calculations (i.e. fully extended conformation) whilst the second preference resembled that found for GABA in the crystal lattice (i.e. partially folded conformation). Proton magnetic resonance studies have favoured the extended conformation of GABA in solution (Parry, Jones, Roberts and Ahmed, 1971). Pullman and Berthod (1974) have recently applied quantum mechanical procedures (PCILO and SCF) to calculate the preferred conformations of the isolated GABA molecule. These calculations (see Pullman and Berthod, 1974) suggest that partially folded conformations of GABA are the energetically favourable ones.

The SARs of two conformationally restricted analogues of GABA have also been investigated. Muscimol, an isoxazole betaine, depresses the firing rate of spinal neurones (Johnston, Curtis, de Groat and Duggan, 1968) and this depressant action is reduced by the GABA antagonist bicuculline (Curtis, Duggan, Felix and Johnston, 1971), hence muscimol probably exerts its effect by an interaction with the same receptors that subserve the depressant action of GABA. Muscimol has been classified as "GABA-like" (see Section III) on the basis of its structural resemblance to GABA, the sensitivity of its depressant action to bicucilline, and because the glycine antagonist, strychnine, in concentrations sufficient to block the depressant action of glycine does not antagonise the depressant action of muscimol (Johnston, Curtis, de Groat and Duggan, 1968). (Strychnine and bicuculline are discussed later in Section I.) Muscimol and GABA have similar potencies as neuronal depressants (Johnston, Curtis, de Groat and Duggan, 1968), and there is good agreement between the crystal conformation data for muscimol (Brehm, Hjeds and Krogsgaard-Larsen, 1972) and GABA (Warner, Player and Steward, 1973) (both conformations being partially folded), and the GABA structure predicted by molecular orbital calculations (Kier and Truit, 1970). The bicuculline-sensitive depressant activity of 4-aminotetrolic acid (4-aminobut-2-ynoic acid) (Beart, Curtis and Johnston, 1971), however, suggests that the active conformation of GABA is an extended conformer. Within the 4-aminotetrolic acid molecule the linear demands of the triple bond prevent the

molecule from folding, and fix the four carbon atoms such that the charged centres are separated by 5.2 to 5.8 Å. 4-Aminotetrolic acid is less potent than GABA, but there could be several features of the molecular structure other than the extended conformation, that are responsible for the potency being less than that of GABA. Thus although the SARs of 4-aminotetrolic acid provided evidence for the extended conformation of GABA being the active conformer there is no agreement amongst the available evidence for GABA and its analogues on this point, and additional compounds, particularly of restricted conformation, need to be investigated. Additional evidence has also been obtained from SAR studies involving GABA antagonists.

The use of antagonists to analyse receptors

Although antagonists of peripheral neurotransmitters have been known and extensively studied for many years, it is only in recent years that specific and effective antagonists of putative central transmitters have been discovered. A pharmacological and physiological investigation of any nervous system must be severely limited if suitable antagonists are not available to provide additional evidence regarding the identity of a transmitter involved in a particular pathway, to provide SAR information, and to provide information on the mechanism of action of centrally acting compounds. For these purposes a suitable antagonist must display a high degree of substrate and process selectivity, and reversibility. The ideal antagonist should interfere reversibly with one of the following transmission processes:

transmitter synthesis, release, postsynaptic action and inactivation. In theory there are other mechanisms of interference with the transmission process, but it is unlikely that they would be specific and hence of pharmacological value.

There are few substances which will selectively interfere with the synthesis or release of a particular transmitter, and thus there have been few investigations of this type conducted in the CNS. Electrophoretically administered hemicholinium III, however, has been shown to selectively block the cholinergic synaptic excitation of Renshaw cells in the feline spinal cord (Quastel and Curtis, 1965), apparently by a progressive reduction in the amount of transmitter release. Recent experiments with tetanus toxin have demonstrated that it reduces the release of both glycine and GABA from electrically stimulated synaptosomal preparations isolated from spinal tissue of mice treated with tetanus toxin (Osborne, Bradford and Jones, 1973), and in vivo experiments indicated that microinjected tetanus toxin reduces the synaptic release of both GABA and glycine (Curtis, Felix, Game and McCulloch, 1973). Hence this toxin would be of no use in distinguishing between GABA releasing and glycine releasing terminals.

Attempts at selectively antagonising the post-synaptic action of putative transmitters have been more successful than the attempts to antagonise synthesis or release, and in particular, microelectrophoretic

experiments utilising post-synaptic antagonists of glycine and GABA have provided valuable pharmacological information for the tentative identification of transmitters and for SAR studies.

Microelectrophoretic considerations

In the previous discussion of the microelectrophoretic administration of agonists it was noted that a neurone under test was subjected to a non-uniform distribution of an ejected agent. This non-uniform distribution raises several difficulties in determining the degree of selectivity and the type of antagonism displayed by a potential antagonist, for the classical sigmoid dose-response curves are derived under conditions where a finite number of receptors are exposed to uniformly distributed agonists and antagonists. Thus the parallel shift in equilibrium dose-response curves due to successive increases in the rate of ejection of an antagonist, may be explained on the basis of a different population of receptors being occupied by the antagonist, compared with the population that is accessible to the ejected agonist. These two populations may be functionally the same, but they have different distribution patterns. For this reason an agonist may appear to surmount the influence of an antagonist because with increasing rates of ejection it gains access to more receptors remote from the ejecting micropipette which are unaffected by the antagonist, and a dose-response curve may then be displaced in a parallel fashion even in the presence of a non-competitive

antagonist, until all receptors on a neurone are affected by the agonist (see Curtis, Duggan and Johnston, 1971). A further complication arising from increasing the rate of ejection of a non-uniformly distributed antagonist is that the concentration of the antagonist may reach relatively high levels at membrane areas close to the micropipette orifice at which non-specific effects of the antagonist begin to complicate the agonist-antagonist interaction. Obviously, with such non-uniform distribution of agents, the location of the relevant receptors on a particular neurone is of importance in determining agonist-antagonist interactions. Thus an agonist may appear to be unaffected by another substance because the accessible receptors for the agonist lie beyond the volume of tissue affected by the potential antagonist, and increasing the rate of ejection of the potential antagonist could, as previously noted, produce non-specific effects at the membrane regions of relatively high antagonist concentrations. In comparing the sensitivity of different agonists to a given antagonist it is at present necessary to assume that the appropriate receptors are similarly distributed in relation to the orifices of the multibarrel micropipette from which the compounds are ejected.

It would seem likely that some of the problems of studying agonist-antagonist interactions in the CNS could be overcome by systemic administration of the antagonist. Many antagonist, however, do not readily penetrate blood-brain barriers. Furthermore, after systemic administration

of an antagonist the concentration in the extracellular environment of a neurone is not accurately known, unless it is measured, although an approximate concentration can be determined by assuming uniform distribution throughout total body water. There are technical and other limitations on the amount of a substance that can be given systemically. For example, if an antagonist of inhibitory transmitters is administered systemically, the firing behaviour and excitability of a neurone under test could be altered by changes in synaptic inputs to the neurone, and hence a limit is imposed on the amount of antagonist that can be systemically administered. Under certain conditions the maximum concentration of antagonist attained by systemic administration may not be sufficient to antagonise the localised concentration of an electrophoretically administered agonist, although antagonism is demonstrated when they are both ejected from a multibarrel micropipette. A somewhat related concentration problem is that which may be observed when an electrophoretically administered antagonist does not attain sufficient local concentration, and distribution parameters, to influence a particular synaptic input to a neurone that is under investigation. In such a case systemic administration may achieve the required concentration and distribution, but complex changes brought about in the pathway being tested or in related pathways could prevent a definitive result being obtained.

Hill and Simmonds (1973) have proposed the use of time-response curves as an alternative to dose-response curves constructed from equilibrium responses. From what has been discussed above regarding the microelectrophoretic administration of agonists and antagonists it is quite clear that there is a complex relationship between the rate of drug ejection and the time course of its concentration at any one point in the tissue, and equilibrium conditions may take many seconds to be established (Curtis, Perrin and Watkins, 1960). It is thus difficult to interpret and compare non-equilibrium type responses, the magnitude of such a response being critically dependent upon the time of ejection. If sufficient time is allowed, the rate of ejection of a substance from a micropipette becomes constant and a steady state concentration gradient develops from the tip of the micropipette in a radial direction. Under these equilibrium conditions the concentration at a given distance from the orifice of the micropipette is directly related to the ejecting current, and inversely related to the distance. The distance is of minor importance when comparisons are made of the relative potencies of substances, ejected from the adjacent barrels of a multibarrel micropipette, on the one neurone, provided the substances interact with the same receptors. In the absence of a direct measure of concentration, the ejecting current must be used to compare the electrophoretically administered 'doses'. Steady state responses are approximately constant, readily reproducible and maximum for a given rate of ejection. Comparison of non-equilibrium

responses can produce apparent differences in potency that are not observed with equilibrium responses. Such apparent potency differences may reflect different rates of increase of concentration at the receptor sites, a variable that needs to be dissociated from estimations of relative potency.

The rate of onset of the effects of an excitant or depressant is invariably reduced when an appropriate antagonist is simultaneously administered. This reduced rate of onset, which may in part be explained by the additional time required for an agonist to attain an effective concentration at receptor sites distal to those blocked by the antagonist, has been employed by Hill, Simmonds and Straughan (1973a,b) to compare the effectiveness of depressant amino acid antagonists. Their method entails estimating the time (T_{50}), from a time-response curve, for a control ejection of an agonist to depress the firing rate of a neurone to 50%, and any change in this response which is subsequently caused by an ejected antagonist is then expressed as a proportion of the control T_{50} value. The change in the T_{50} value is said to represent a displacement of the time-response curve and hence can be used to compare the effectiveness of depressant amino acid antagonists (see Hill and Simmonds, 1973; Simmonds, 1974). This method of comparison appears to offer no advantage over that based on a comparison of equilibrium responses, a method which does not make use of parameters that are derived directly from the complex, and poorly understood,

initial phase of a response to an electrophoretically administered agent. Furthermore, some of the conclusions drawn from comparisons using time-response curves appear to be at variance with the findings of other investigators. For example, the reports that bicuculline is a weak GABA antagonist, capable of 'potentiating' GABA, (Hill and Simmonds, 1973; Hill, Simmonds and Straughan, 1973b; Straughan, Neal, Simmonds, Collins and Hill, 1971) can be contrasted with the numerous reports quoted by Curtis and Johnston (1974b) that bicuculline selectively antagonises GABA, and synaptic inhibitions believed to be mediated by GABA, in various regions of the feline CNS.

That the microelectrophoretic method has produced results that are apparently at variance is indicative of the technical and interpretational difficulties of this complicated technique which has perhaps been too readily undertaken by those unaware of its complexities. As discussed above, the method is at present not amenable to exact quantitation, and the results of investigations utilising microelectrophoresis must be viewed in this light. Unfortunately, the investigation of SARs, which is essential for expansion of pharmacological information concerning the CNS, necessitates a quantitative approach. The application of microelectrophoresis, in its present form, to SAR studies is time consuming, and it is obvious that the problems that need to be solved before the technique can be rendered more quantitative will in themselves require considerable time, concentrated effort and acquired

expertise. In the meantime, a compromise needs to be made between quantitative reliability and the demand for structure-activity data, even if it is of a semi-quantitative nature.

Structure-activity relationships involving antagonists

SAR studies that are similar to those described for agonists have also been carried out for antagonists as a means of determining the macromolecular structure and function of the sites at which agonists and antagonists interact. Again, systematic structural variation accompanied by biological testing can reveal trends which influence activity, if it is assumed that receptor affinity depends upon specific structural features which are common to both agonist and antagonist molecules. It is possible, however, to note examples of antagonists that have only a distant structural relationship to the parent agonist molecule. For example, the rather diverse collection of molecular structures which have been reported to be antagonists of noradrenergic α-receptor mediated responses could not all be expected to relate to the site of catecholamine interaction at the α-receptor. It is highly probable that their SARs refer to some other site(s) at which modulation of some stage(s) in the post receptor-agonist interaction sequence occurs. Monod, Changeaux and Jacob (1963) have indicated in their discussion of allosteric proteins that the concept of competitive interaction between agonist and antagonist does not

necessarily imply physical competition for the same binding site. Hence an antagonist could either directly, or allosterically influence agonist-receptor interaction, or a subsequent process, and the number of potential sites of antagonist interaction could thus be quite large. For this reason an investigation of the SARs of antagonists that are not specific or do not have a common and obvious structural resemblance to the parent agonist may be of doubtful value in providing information about the agonist receptor.

Specific antagonists of the inhibitory amino acid transmitters, GABA and glycine, have been discovered, and some SAR data for these antagonists are available.

The GABA receptor

Bicuculline and picrotoxinin have been accepted as central GABA antagonists (see Curtis and Johnston, 1974a, b; Krnjević, 1974 and Section IV), and, unlike GABA and its active analogues, the molecules of these two antagonists are relatively inflexible. Hence it may thus be easier to identify a common three dimensional pattern of functional groups required for interaction with GABA receptors. Data are available on the crystal structures of bicuculline and picrotoxinin, and in addition, molecular orbital calculations have been performed for the bicuculline molecule, although the relative rigidity of this molecule tends to ensure that the crystal data and the molecular orbital calculations produce similar results.

It would be expected that picrotoxinin and bicuculline have a charged cationic centre at a certain distance from a carboxyl anionic centre or a lactone ring in order to combine with the GABA receptor. The cationic centre in the bicuculline molecule is the nitrogen atom, and two of the three methyl groups in the picrotoxinin molecule are likely to be partially charged. Craven (1962) has reported that in the crystal structure of α1-picrotoxinin hydrobromide one methyl group participates in hydrogen bonding to the bromine atom, whilst Jerslev and co-workers (Jerslev, Ravn-Jonsen and Danielsen, 1968) have reported that in β-picrotoxinin hydrobromide a second methyl group forms a similar hydrogen bond. Thus with the two lactone rings there is a total of four possible charge arrangements of importance in the picrotoxinin molecule. A close agreement has been reported between the crystal structures of GABA, muscimol and bicuculline (Warner, Player and Steward, 1973), indicating that they may all adopt conformations giving similar distributions of charge centres.

It was suggested, from a study of Drieding stereo-models of GABA, muscimol and bicuculline, that the nitrogen atoms and the three atom grouping of the carboxylate in GABA (O=C-O) in an extended conformation, or the equivalent in muscimol (N=C-O), can be exactly isosteric with the nitrogen atom and the O=C-C grouping in bicuculline (Curtis, Duggan, Felix and Johnston, 1970; Johnston and Curtis, 1973). In a similar manner it has been shown that when the carboxylate group of 4-amino-

tetrolic acid is superimposed on the C-C=O grouping in bicuculline the nitrogen atoms in each molecule can be within 0.5 Å of each other (Beart, Curtis and Johnston, 1971). An alternative model of GABA-bicuculline antagonism (Steward, Player, Quilliam, Brown and Pringle, 1971; Steward, Player and Warner, 1973) is based on the carboxylate group of a partially folded conformation of GABA being isosteric with the O-C=O grouping in bicuculline, but 4-aminotetrolic acid cannot assume the folded conformation required by this model.

It is apparent that a large number of GABA antagonists and agonists need to be considered before any significant SARs may emerge, and at this stage it seems to be too early to speculate on a 'recognition' conformation that is available to all active GABA analogues and antagonists, and a second 'active' conformation that is only available to GABA agonists (Warner, Player and Steward, 1973). Despite the early stage of development of GABA SARs, Smythies (1971, 1974) has developed an hypothetical three dimensional GABA receptor from a knowledge of the molecular structure of GABA, picrotoxinin and bicuculline, and the model has successfully predicted, on the basis of molecular complementarity to the simple protein structure of the receptor, that a number of known convulsant poisons act by competitive antagonism at the GABA receptor.

The glycine receptor

At a number of sites within the mammalian CNS strychnine is a reversible and selective antagonist of the inhibitory action of glycine (see Curtis and Johnston, 1974a, b), and the antagonism appears to be competitive in type (Johnson, Roberts and Straughan, 1970; Curtis, Duggan and Johnston, 1971). A very large number of compounds have been shown to suppress spinal inhibition of the strychnine sensitive type, and to antagonise the effects of glycine when administered electrophoretically. The structures of these glycine antagonists vary considerably from strychnine-like, such as brucine (Curtis, Hösli and Johnston, 1968), to bicuculline-like, such as laudanosine (Curtis, Duggan, Felix and Johnston, 1971), or picrotoxinin-like, such as dendrobine (Curtis, Duggan, Felix and Johnston, 1971). Because of the wide variety of structures that display the property of glycine antagonism it is difficult to identify common conformational elements that may be responsible for this property, and hence knowledge of these structures has not helped greatly in analysing the glycine receptor. It is conceivable that the glycine antagonists may act at several different sites either at or in the vicinity of the glycine receptor. Nevertheless, Smythies (1971, 1974) has used the structures of glycine and strychnine to construct an hypothetical glycine receptor which accommodates many of the known glycine antagonists, and predicts that several compounds owe their convulsant activities to blockade of the

glycine receptor. Smythies hypothesis speculates on the actual three dimensional nature of the glycine receptor, and as such it can only be tested by satisfying the available SAR data and predicting the activity of new compounds.

In view of the apparently diverse molecular structures that display glycine antagonism it would seem that a profitable line of approach may be the extension of the work by Young and Snyder (1973), who have reported the binding of tritiated strychnine to membrane fractions of rat brain stem and spinal cord tissue. The binding was competitive with glycine-like amino acids, and of the subcellular fractions examined, the highest specific strychnine binding activity occurred in the synaptic-membrane fraction, suggesting that the binding sites were concentrated on postsynaptic glycine receptors. Extension of this work may lead to isolation of the glycine 'receptor', in which case a direct examination of the 'receptor' could possibly be undertaken.

Antagonism of transmitter inactivation

Synaptically released transmitters or electrophoretically administered putative transmitters and their analogues could be removed from the receptor environment by diffusion, extracellular enzymic modification, and binding to or uptake by surrounding tissue components. The electrophoretic administration of hydrazides, hydroxylamine and amino-oxyacetic acid (Curtis, Hösli and Johnston, 1968; Curtis, Duggan and Johnston, 1970;

Steiner, 1969; Gottesfeld, Kelly and Renaud, 1972), all of which are inhibitors of enzymes that are likely to be involved in the degradation of amino acid transmitters, has not produced evidence of the selective modification of amino acid or transmitter action. Systemic administration of these agents may produce changes that cannot be interpreted solely in terms of effects on extracellular amino acid inactivation. On the other hand, the inhibitory action of GABA and glycine on the firing rates of feline spinal neurones is potentiated by p-chloromercuriphenylsulphonate, and higher concentrations of this mercurial also potentiate the excitation of these neurones by L-glutamate and L-aspartate (Curtis, Duggan and Johnston, 1970). This mercurial has been shown to inhibit the active transport of glycine, GABA and DL-aspartate into rat brain slices (Curtis, Duggan and Johnston, 1970), and hence it is likely that active transport is an important factor in terminating the actions of amino acids in the extraneuronal environment. If carrier-mediated cellular uptake is responsible for limiting the immediate action of amino acids in the subsynaptic region (Iversen and Neal, 1968; Curtis, Duggan and Johnston, 1970; Snyder, Logan, Bennett and Arregui, 1973), then the uptake receptor is of considerable interest in that it is a site for potential modification of synaptic processes. Selective antagonism at the uptake site would be expected to prolong and enhance the action of electrophoretically administered putative transmitters and the appropriate synaptic processes, thus providing evidence concerning the identification of a transmitter. Clearly, for such an uptake antagonist to be developed it is necessary to determine whether the

receptors that subserve excitation or depression are structurally different from those for uptake.

Uptake processes are most readily investigated *in vitro* using tissue slices and subcellular particulate preparations which actively transport amino acids. These preparations allow a study of the kinetics and substrate specificity of the processes, and the effects of antagonists, but due consideration must be given to regional and species differences in the transport of amino acids. CNS tissue has complex metabolic, ionic and organisational requirements, and the transport of amino acids by CNS tissue preparations *in vitro* must represent the accumulation of amino acids by glial and neuronal elements under abnormal metabolic and ionic conditions. The results obtained *in vitro* may thus not be strictly relevant to conditions *in vivo* in the vicinity of synapses. Although a large number of compounds have been reported to inhibit the uptake of amino acids *in vitro*, few are suitable for use for specific modification of amino acid or transmitter inactivition *in vivo*.

Hydrazinoacetic acid and chlorpromazine have been reported to inhibit the specific high affinity uptake of glycine by rat spinal cord slices *in vitro* (Iversen and Johnston, 1971) and cat spinal cord slices *in vitro* (Balcar and Johnston, 1973), but both substances have direct depressant actions on spinal neurones, and no glycine analogues have been found to enhance the inhibitory action of glycine (Curtis, Hösli and Johnston, 1968). Various mercurials, for example, *p*-chloromercuriphenyl-sulphonate, which was mentioned

above, will potentiate the inhibitory effect of glycine, but the potentiation is relatively non-specific (Curtis, Hösli and Johnston, 1968; Curtis, Duggan and Johnston, 1970) and reflects the non-specific inhibition of transport processes that is seen *in vitro* (Balcar and Johnston, 1973). Strychnine does not inhibit glycine uptake by cat spinal cord slices *in vitro* (Balcar and Johnston, 1973). Similarly, there are differences between the GABA postsynaptic receptor and the GABA uptake receptor - bicuculline does not inhibit the uptake of GABA by cat spinal cord slices (Balcar and Johnston, 1973) and neither picrotoxinin nor bicuculline inhibits GABA uptake by rat brain slices (Curtis, Duggan, Felix and Johnston, 1970; Iversen and Johnston, 1971; Johnston and Mitchel, 1971). A number of GABA analogues inhibit the uptake of GABA by nervous tissue *in vitro* (Balcar and Johnston, 1973; Beart and Johnston, 1973; Beart, Johnston and Uhr, 1972; Iversen and Johnston, 1971) but none of the most potent inhibitors, which include 4-aminovaleric acid, 2-methyl-GABA, 2-fluoro-GABA, 2-chloro-GABA, 2-methyl-GABA, has yet been reported to be a specific GABA uptake inhibitor without direct depressant effects on neuronal firing.

L-Glutamate and L-aspartate appear to be substrates of comparable affinity for the same high affinity, acidic amino acid uptake system in rat brain (Balcar and Johnston, 1972a), and the proposed three point receptor for the postsynaptic action of excitant amino acids (Curtis and Watkins, 1965) may also apply to the uptake site. Thus derivatives of L-glutamate or L-aspartate in which the polarity of any of the three charged centres was modified

are without effect on glutamate or aspartate uptake (Hammerschlag, Potter and Vinci, 1971; Balcar and Johnston, 1972a,b). Mono-amino-dicarboxylic acids containing a longer carbon chain than glutamate were either weak (DL-3-aminoadipate) or ineffective (DL-2-aminoadipate, DL-2-aminopimelate and 4-aminopimelate) inhibitors of glutamate uptake (Balcar and Johnston, 1972a), indicating that the uptake receptor has certain steric requirements. Several potent amino acid excitants (N-methyl-D-aspartate, D-homocysteate and N-ethyl-D-aspartate) have no influence on glutamate uptake (Balcar and Johnston, 1972b), and hence the uptake receptor presumably displays structural requirements that are different from those of the postsynaptic receptor. (This observation emphasises the problem of comparing the potencies of excitants which may be treated differently by the acidic amino acid transport system.) Ibotenic acid, a relatively rigid and extended conformation of glutamate, did not inhibit glutamate uptake in rat brain slices (Balcar and Johnston, 1972a), suggesting that folded conformations of glutamate may be more important at the uptake site. Antagonists of the excitatory action of glutamate: L-glutamate diethylester, DL-α-methyl glutamic acid (Haldeman, Huffman, Marshall and McLennan, 1972), L-methionine-DL-sulphoximine (Curtis, Duggan, Felix, Johnston, Tebēcis and Watkins, 1972) and lysergic acid diethylamide (Boakes, Bradley, Briggs and Dray, 1970) do not influence glutamate uptake by either rat brain slices or cat spinal cord slices (Balcar and Johnston, 1972a and 1973). No selective inhibitor of either

L-glutamate or L-aspartate uptake has been reported, and amongst the most effective inhibitors of the excitant amino acid uptake system, D-aspartate, threo-3-hydroxy-DL-aspartate, L-α-cysteinesulphinate and L-cysteate are comparable in strength with L-glutamate as excitants of feline spinal neurones (Curtis and Watkins, 1960; Curtis, Duggan, Felix, Johnston, Tebēcis and Watkins, 1972). Thus whilst several compounds will excite neurones directly, without significantly affecting excitant amino acid uptake, a compound has yet to be reported that will selectively inhibit the uptake of glutamate or aspartate and not itself produce excitation.

II. METHODS

The experiments described in this thesis were performed on various *in vivo* preparations of the feline central nervous system. These preparations were used either for the recording of extracellular action potentials of single neurones during the microelectrophoretic or systemic administration of substances, or for the recording of spinal reflexes in order to determine the effects of intravenously administered substances on the inhibition of spinal reflexes.

(1) Feline central nervous system preparations

One of five different types of feline preparation was employed depending upon the aim of the experiment; some methods, however, were common to all of these preparations.

(a) General methods

Cats of either sex were lightly anaesthetised with pentobarbitone sodium (35 mg/kg intraperitoneally initial, supplemented intravenously when required) and following the induction of anaesthesia the left cephalic vein, the right common carotid artery (spinal cord preparation) or left femoral artery (supraspinal preparations) and the trachea were cannulated. After preparation of the recording site and peripheral nerves for stimulation, as described in the following paragraphs, the animal was mounted in a frame of extreme rigidity using thoracic and pelvic clamps

in addition to the head frame (supraspinal preparations) and lumbar clamps (spinal cord preparation). The animal was suspended so that the abdomen and thorax was held clear of the base of the frame to reduce respiratory movement of the cerebral tissue. When necessary, gallamine triethiodide was administered intravenously (4 mg/kg initially, 2 mg/kg half hourly) and the animal artificially respired to maintain an end tidal CO_2 level at 4-4.5%. A bilateral pneumothorax was occasionally performed to further reduce respiratory movement of cerebral tissue. The temperature of all animals was maintained between 36-38^{o}C by means of heating pads placed beneath the abdomen and chest. A thermometer and temperature sensitive probe were placed between the rib cage and right scapula and the current flowing through one of the heating pads was controlled by a solid state current regulating unit. The carotid or femoral blood pressure was continuously monitored.

(b) Spinal cord preparations

The laminae of all lumbar vertebrae were removed and the spinal cord transected at the lower thoracic level after infiltration with 1% procaine solution. The dural sac was opened dorsally over the lumbar and sacral cord, and the seventh lumbar and first sacral ventral roots were divided at the intervertebral foramen and mounted on platinum electrodes which served for either stimulation or recording. In some experiments (Section VIII(2)) the seventh lumbar dorsal root was

also transected, separated into upper and lower portions and mounted on electrodes for stimulation. After mounting the animal in a frame of extreme rigidity, as previously described, the exposed spinal cord and vertebral muscles were covered with warmed liquid paraffin (BP) which was retained by skin flaps.

(i) Spinal interneurone and Renshaw cell preparation

Prior to preparation of the recording site, the left sural, common peroneal and tibial nerves were transected and mounted on platinum stimulating electrodes, the exposed nerves being immersed in warmed paraffin oil. These nerves were stimulated at 3-5 X threshold, a platinum ball electrode placed on the dorsal surface of the cord being used to monitor the resulting afferent volleys and thus to determine nerve thesholds and central latencies of excited internuerones. The ball electrode on the dorsal surface of the cord was also used to record the 'Renshaw ripple' (Eccles, Fatt and Koketsu, 1954) in response to ventral root stimulation. After suitable amplification these potentials were displayed on a cathode ray oscilloscope from which filmed records could be made. Renshaw cells were identified by responses which followed antidromic stimulation of ventral roots. Small areas of pia-mater were carefully removed from the left lower lumbar and upper sacral segments of the dorsal surface of the cord

at the site of proposed electrode penetration. Fine forceps and a dissecting microscope were used to prepare these recording sites.

(ii) Spinal reflex preparation

Monosynaptic reflexes were recorded monophasically from the seventh lumbar (L7) or first sacral (S1) ventral root in response to electrical stimulation (0.5 or 1/sec, maximum intensity for the fastest afferent fibres) of transected hind limb muscle nerves - posterior biceps and semitendinosus (PBST); medial, lateral gastrocnemius and soleus (GS); flexor digitorum longus (FDL); peronei and muscles innervated by the deep peroneal nerve (PDP) and quadriceps (Q). These reflexes were usually faciliated and stabilised by preceding submaximal stimulation of the same nerve, this stimulus being subliminal for a reflex response. Reflexes initiated by stimulation of one muscle nerve were inhibited by volleys in other muscle or cutaneous nerves.

Reflexes were suitably amplified and displayed on a cathode ray oscilloscope. The area beneath the monosynaptic reflex was measured using a voltage to frequency converter (VIDAR 260), and a gated counter with analogue output (Hewlett Packard Counter 5214L and Digital Recorder H23-562A). The recorder output was used to construct inhibitory curves which were plotted directly on an X-Y recorder (Varian F-80,

converted to a point ploter): the X-voltage was proportional to the interval between the inhibitory and testing volleys, the Y-voltage was proportional to the area beneath the monosynaptic reflex.

Dorsal root potentials and dorsal root reflexes were recorded from the most caudal rootlet of the sixth lumbar (L6) dorsal root with an amplifier having a time constant of 1 sec. The 'Renshaw ripple' (see paragraph (I) (b) (i) above) and 'quadriceps notch' (Eccles, Fatt and Landgren, 1956), in response to stimulation of the ventral roots and the left quadriceps nerve respectively, were recorded by a ball electrode on the dorso-lateral surface of the spinal cord. These potentials were suitably amplified and filmed from an oscilloscope display.

(c) Cerebellar cortex preparation

Lobules V and VI of the cerebellar vermis (culmen and lobulus simplex) were exposed by removal of an approximately circular piece of bone (8-10 mm in diameter) just anterior to the lamboid ridge. The underlying dura-mater was sectioned and the exposed cortex continuously flushed with a warmed (38^{o}C) mammalian Ringer solution which was equilibrated with 5% CO_2, 95% O_2. The head was held in a modified Horsley-Clark head frame which allowed rotation about an axis parallel to the interaural line, and thus the area of vermis from which recordings were taken could

be placed in an approximately horizontal plane. After preparation of the recording site the head frame was fixed to the animal frame and the cat suspended as previously described.

An area for recording was chosen that was relatively free from superficial cortical vessels and a small perspex pressor foot was carefully placed upon the tissue to reduce cortical pulsations. No attempt was made to remove the pia-mater, for such removal results in damage to the superficial cell layers and in most cases the micropipettes penetrated the pia without difficulty. A co-axial stimulating electrode (0.5 mm in diameter) was also placed on the surface of the vermis, 1-2 mm medial to the recording site and on the crest of the same folium. Both the pressor foot and the co-axial stimulating electrode were carried on manipulators fixed to the head frame.

Cerebellar Purkinje cells were identified by their depth beneath the cortical surface (150-400 μm), the recording of spontaneous 'inactivation responses' (Granit and Philips, 1956), and the inhibitory response to electrical stimulation of the cortical surface using the co-axial electrode 'off-line' from the recording site (Andersen, Eccles and Voorhoeve, 1964).

(d) Cerebral cortex preparation

The medullary pyramids were exposed from the ventral aspect by partial removal of the basi-occiput, and the head of the animal was then fixed in a modified

Horsley-Clark head frame which was attached to the frame in which the cat was suspended, and allowed the pericruciate cortex to be orientated in an approximately horizontal plane. The left pre- and postcruciate cortical areas were exposed by removal of the overlying bone and the posterior wall of the left frontal sinus. After opening the dura the cortex was covered with polyethylene sheeting, apart from a small opening left at the recording site. During microscopic examination of the cortex the pia-mater was gently removed with fine forceps from suitable small, superficially avascular, areas. Throughout the experiment the whole area was irrigated with warmed (38^{o}C) mammalian Ringer solution which was equilibrated with 5% CO_2, 95% O_2. A small perspex pressor foot plate, which was carried on a micromanipulator fixed to the head frame, was used to stabilise the cortex at the recording site.

Deep pyramidal cells of the postcruciate cortex were identified by a constant latency antidromic response to electrical stimulation of the exposed ipsilateral medullary pyramid (latency within the range 0.5-5 msec), and by the ability to follow tetanic antidromic volleys (see Philips, 1956, 1959, 1961).

(e) Ventrobasal thalamus preparation

The right median and superficial radial nerves were transected and mounted on platinum stimulating electrodes in a warmed paraffin pool formed by skin flaps. With the head of the animal held in a modified

Horsley-Clark head frame, the left cranial vault was removed and the dorsal surface of the thalamus exposed by partial removal of the overlying cerebral cortex. The exposed thalamus and surrounding brain were continuously irrigated with mammalian Ringer solution (38^{o}C, equilibrated with 5% CO_2 and 95% O_2) and the head frame was fixed to the rigid frame which suspended the animal as previously described.

Thalamic neurones were located stereotaxically by passing micropipettes in a vertical plane, using the co-ordinates published by Jasper and Ajmone-Marsan (1954). Thalamo-cortical relay neurones were located in the region of the left nucleus ventralis posterio-lateralis and nucleus ventralis posteriomedialis and were identified on the basis of short latency (6-8 msec) responses to impulses in one contralateral peripheral cutaneous nerve of the forelimb.

(2) Microelectrophoresis

The technique of microelectrophoresis was used to determine the pharmacological effect of substances on individual neurones. Ionised compounds were electrically ejected into the extraneuronal space from multibarrel glass micropipettes whilst simultaneously recording the extracellular action potentials of neurones adjacent to the tip of the micropipette. The methods used were those described by Curtis (1964). Seven barrel micropipette 'blanks' were drawn out to a fine tip in a vertical electrode puller. These very fine tips were viewed

microscopically, and, by means of a fine glass rod attached to a micromanipulator (de Fonbrune No. 190), the tips were broken back to a total diameter of 5-8 μm. The dry micropipettes were then filled from above with centrifuged solutions and filling was completed by centrifuging the partially filled micropipette. The centre barrel of each micropipette was filled with 4 M NaCl, and the six outer barrels contained aqueous solutions of the substances to be tested, in the particular combination required for an experiment. Details of the solutions of standard excitants, depressants and antagonists are contained in Table 1, and details of the other microelectrophoretic solutions are given in the appropriate results sections.

TABLE 1 Standard solutions for microelectrophoresis

Compound	Molarity	pH	Acid/alkali added
Acetylcholine bromide	0.5	-	-
γ-Aminobutyric acid (GABA)	0.5	3	HCl
L-Aspartic acid	1.0	8	NaOH
(+)-Bicuculline methochloride (BMC)	0.005*	3	HCl
(+)-Bicuculline hydrochloride	0.010*	3.5	-
	or 0.100		-
L-Glutamic acid	1.0	8	NaOH
Glycine	0.5	3	HCl
DL-Homocysteic acid (DLH)	0.2	7.5	NaOH
Noradrenaline bitartrate	0.1	3.6	-
Strychnine hydrochloride	0.002*		
	or 0.01*		

* in 165 mM NaCl

After filling, the micropipettes were examined microscopically using a water immersion lens and the electrical resistance of each barrel was measured. Micropipettes were also examined microscopically after use. The micropipettes were carried on a micromanipulator which allowed movement in three axes (Eccles, Fatt, Landgren and Winsbury, 1954), and which was attached to the rigid animal frame. The vertical movement of this micromanipulator was occasionally replaced by a remotely controlled micromanipulator (Eide and Källström, 1968; Stålex Micromanipulator, type Lundberg). When the microelectrode was placed in the tissue, the resistance of each barrel was again measured. Electrical contact was made with the centre (recording) barrel by means of a small Ag/AgCl junction, and with the other barrels by means of a silver wire. An Ag/AgCl junction, covered with gauze saturated with 165 mM NaCl, was sutured to exposed muscle near the recording site to act as the indifferent electrode (earth). The resistance of each barrel in situ was determined by establishing a 0.5 V potential difference across each solution and measuring the resulting current flow. Retaining potentials of the order of 0.5 V were applied to each outer barrel in order to control the diffusional efflux of active ions, the resulting retaining current was maintained throughout the experiment. Currents ejecting cations have been termed cationic (+), whilst those ejecting anions have been termed anionic (-). Ejecting and retaining currents have been quoted in nano-amperes (nA, 10^{-9} A) and were measured with an accuracy of ± 0.5 nA.

(a) The recording of extracellular action potentials

The Ag/AgCl junction which made electrical connection with the recording barrel of the micropipette was connected to a negative capacitance cathode follower. After suitable amplification the spike potentials were displayed upon a double beam oscilloscope from which photographic records could be made. In addition, the output of the cathode follower was also amplified and used to trigger a pulse generator. The output pulses of this generator and the spike potentials were observed simultaneously on the double beam oscilloscope, and a voltage discriminator was used to select the size of the action potential that triggered the pulse generator. The pulses so generated were counted by means of a rate meter and the output of the rate meter (time constant 0.2 sec) was displayed on a rectilinear pen recorder (Texas Instruments Recti-Riter) upon which side marker pens indicated periods of microelectrophoretic ejection of compounds. There was thus a continuous display of mean cell firing frequency, and this could be readily correlated with the effects of electrophoretically administered compounds. The number of Renshaw cell action potentials in response to ventral root stimulation were counted using a gated counter and digital recorder (Hewlett Packard Counter 5214L and Digital Recorder H23-562A).

(b) Experimental design

The neurones studied were either firing spontaneously, in response to electrophoretic pulses of excitants, or were excited by the continuous electrophoretic administration of an excitant. The current ejecting the excitant was adjusted, if necessary, to maintain a constant firing frequency despite changes in the excitability of neurones brought about by the administration of other substances (e.g. bicuculline). The following procedures were adopted for specific experiments.

(i) The depressant activity of GABA analogues

Neurones were selected that were sensitive to the depressant action of GABA, and the responses to electrophoretically ejected test substances were compared with those to GABA and glycine. The potency of a test substance relative to the potency of GABA or glycine was obtained by determining the equipotent current ratio, the ratio of ejecting currents which produced equal, submaximal and steady state firing rates when the substances to be compared were ejected from the same micropipette. The latencies of onset and offset of responses to depressants could not be accurately compared from the rate meter recording of the firing frequency, unless these times exceeded 2-5 seconds. In some experiments photographic records were used to determine offset times. The criteria of Curtis and Koizumi (1961)

were used to identify current effects from true depression, and with weak depressants it was often necessary to include a current control barrel (165 mM NaCl) in the micropipette, in addition to DL-homocysteate (DLH), GABA, glycine and the one or two barrels that contained the analogues to be tested. (See Table I for details of the standard solutions and Section III for details of the GABA analogue solutions.)

β-(p-Chlorophenyl)-GABA was also administered intravenously as a 1 mg/kg solution in 165 mM NaCl to determine the effect of this compound on spinal reflexes, DRPs, the 'quadriceps notch' and the 'Renshaw ripple'.

(ii) GABA and glycine antagonism or potentiation

Currents ejecting glycine and GABA were chosen to produce just maximal or submaximal depression of firing when equilibrium firing rates were established (usually within 10 sec), and these responses were used to determine the effectiveness of a test substance as a glycine or GABA antagonist. The test substance was continuously administered for a period judged to be sufficient to allow equilibrium conditions to be developed. The specificity of antagonism was determined by the differential effect on GABA and glycine responses, and any observed antagonism was compared with that due to known antagonists - bicuculline for GABA antagonism

and strychnine for glycine antagonism. Some substances which were tested electrophoretically as GABA or glycine antagonists were also tested for their effects on the inhibition of spinal reflexes after intravenous administration.

The following substances, dissolved in 165 mM NaCl, were administered intravenously for testing on the inhibition of spinal reflexes: corlumine (1 mg/ml), benzylpenicillin sodium (0.18 g/ml: approximately 0.3 mega units/ml), shikimin (1 mg/ml), strychnine hydrochloride (0.1 mg/ml) and tutin (0.5 mg/ml). (The significance of a reduction in spinal 'direct' or 'presynaptic' inhibitions after systemic administration of a convulsant substance is outlined in the discussion to Section IV).

A similar experimental design was used to determine the potentiation of glycine or GABA depressant responses by an electrophoretically administered test substance, however, the control glycine and GABA responses were kept at a magnitude which would allow for significant potentiation to be recorded.

The outer barrels of the micropipettes used in these experiments usually contained the following combination of solutions (for details of the standard solutions see Table 1): DLH, GABA, glycine, bicuculline (or bicuculline methochloride), strychnine hydrochloride and one of the

compounds to be investigated as an antagonist (for details of the latter solutions see Section IV or V).

(iii) Anticholinesterase activity of bicuculline hydrochloride and bicuculline methochloride (BMC)

Either bicuculline hydrochloride or BMC was used to antagonise the inhibitory action of GABA on Renshaw cells that were being excited by the continuous administration of DLH. The current of bicuculline hydrochloride or BMC that was thus demonstrated to significantly antagonise electrophoretically ejected GABA was administered again whilst the cell was excited by alternate electrophoretic pulses of DLH and acetylcholine. Potentiation of the acetylcholine response could be gauged by comparison of control responses with those during the administration of BMC or bicuculline hydrochloride.

The outer barrels of the micropipettes used in these experiments contained the following solutions: DLH, acetylcholine, GABA, glycine, BMC, bicuculline hydrochloride (see Table 1 for details).

The effect was also determined of intravenously administered physostigmine salicylate (0.1 mg/ml 165 mM NaCl) and bicuculline hydrochloride (0.1 mg free base/ml 165 mM NaCl) on the responses of

Renshaw cells to electrophoretically administered excitants or to stimulation of the ventral roots.

(iv) Relative potency of excitants and relative sensitivity of neurones to excitants

The relative potencies of excitants were obtained by determining equipotent current ratios, the ratio of ejecting currents (from the same micropipette) which produced equal, submaximal and steady state firing rates, the more potent excitant being effective with the lower ejecting current. The reciprocal of the equipotent current ratio was taken as the sensitivity ratio for any one neurone. This method assumes that the agents are interacting with the same receptor, and that the active concentrations produced at the neuronal membrane are directly proportional to the electrophoretic current. (These assumptions are discussed in Section 1). Despite the necessary assumptions, the method is preferable to a comparison of effects produced by 'equal' extraneuronal concentrations and thus a determination of potency ratios which depend upon the portions of the dose-response curves which are being studied (compare Crawford and Curtis, 1964; Krnjević and Phillis, 1963. p.282).

Kainic and N-methyl-D-aspartic acids were found to be potent neuronal excitants, and on structural grounds were considered to be potentially specific for glutamate and aspartate

receptors, respectively (see Sections VI and VII). It was thus decided to use these two excitants to determine the relative sensitivity of interneurones and Renshaw cells to two excitants that might be relatively more specific for glutamate and aspartate receptors than the parent amino acids. Both kainate and NMDA (N-methyl-D-aspartate) were potent neuronal excitants when administered as anions from 0.1 or 0.2 M solutions, and excitation frequently occurred with mere reduction of the cationic retaining currents. The relative sensitivity of neurones, or the relative potencies of agonists cannot be accurately obtained from responses that are produced by reduced retaining currents. In such cases the amount of agonist that leaves the micropipette need not be directly related to the reduction in retaining current, and furthermore, the zero 'dose' level on the retaining scale is not accurately known. Similarly, accurate comparisons of potencies or sensitivities cannot be made from responses produced by removal of the retaining potential. When the retaining potential is removed free diffusion occurs, and although a response may be observed, no measure of the 'dose' of agonist is possible, and the resulting rate of diffusion of substances from different micropipette barrels would depend upon a large number of variables. Thus, in order to compare accurately the responses to kainate and NMDA, it was necessary for these excitants to be administered with anionic currents that were readily measured.

Preliminary experiments established that approximately equal anionic ejecting currents (within the range 20-80 nA) were adequate to excite spinal neurones submaximally when solutions of kainate and NMDA were diluted with NaCl (kainate 5 mM in 250 mM NaCl; NMDA, 50 mM in 200 mM NaCl). The fraction of the electrophoretic current ejecting the amino acid anions from dilute solutions in NaCl was assumed to be 1/51 of that passed through the kainate barrel, and 1/5 of that through the NMDA barrel. The sensitivity ratios were thus corrected for this dilution factor of IO. To minimise any errors due to comparisons being made with different micropipettes an approximately equal number of Renshaw cells and interneurones were tested with any one micro-pipette.

The excitants that were to be compared were contained in the outer barrels of micropipettes as follows: DLH, L-glutamate, L-aspartate or 'cycloglutamate' (1 M, adjusted to pH 7.3 with NaOH), ibotenate (0.1 M, pH 7, NaOH) or dihydrokainate (0.2 M, pH 7, NaOH), NMDA (0.1 M, pH 8) and kainate (0.1 M, pH 8, NaOH). Those micropipettes that were used to determine the relative sensitivity of interneurones and Renshaw cells contained solutions of NMDA and kainate that were diluted with NaCl as described above. (For details of the standard solutions see Table 1).

(v) Glutamate antagonism

The ability of an electrophoretically administered substance to antagonise the excitation of neurones by L-glutamic acid was tested on either spinal interneurones or Renshaw cells, except for I-hydroxy-3-aminopyrrolidone-2 (HAP) which was also tested on acetylcholine sensitive neurones of cerebral cortex and ventrobasal thalamus. The test substance was examined for depressant/antagonistic activity on neurones that were excited by L-glutamate. Specificity of antagonism was determined by testing the proposed antagonist on both glutamate and aspartate mediated excitation, and in the case of acetylcholine sensitive neurones it was also possible to test the effect of substances on acetylcholine mediated excitation. Reduction of glutamate and acetylcholine, or aspartate and acetylcholine mediated excitations was termed 'non-specific' depression. A further indication of 'non-specific' depression was reduction in spike height during the administration of the test substance, an indication of a local anaesthetic-like effect (Curtis and Phillis, 1960). When Renshaw cells were used to test for glutamate antagonism, the effect of the test substance on the synaptic excitation of these cells by stimulation of the ventral and dorsal roots could also be examined. Reduction of the Renshaw cell submaximal ventral root response was an additional indication of 'non-specific' depression. The testing procedures

that were employed also permitted the detection of excitation of neurones by test substances.

The following combination of solutions was contained in the outer barrels of micropipettes used in these experiments: L-glutamate, L-aspartate, DLH, acetylcholine and two of the substances to be tested as glutamate antagonists (for details of the standard solutions see Table 1, the details of the other solutions are contained in Table 7).

(3) Sources of drugs and chemicals

(a) Provided by:

Dr W.L.F. Armarego, Canberra:

(±)-cis-2-aminocyclohexane-1-carboxylic acid

(±) trans-2-aminocyclohexane-1-carboxylic acid

Dr R.B. B-Johns, Melbourne:

Coriamyrtin

CIBA

β-(p-chlorophenyl)-γ-aminobutyric acid

Dr C.H. Eugster, Zurich:

Ibotenic acid

Professor F. Fastier, Otago:

Tutin

Dr F.R. Hewgill, Nedlands:

(±) trans-3-aminocyclohexane-1-carboxylic acid

Professor Y. Kajimoto, Tokushima:

Shikimin

Dr D.I.B. Kerr, Adelaide:

(±)-cis-3-aminocyclohexane-1-carboxylic acid

Dr P. Krogsgård-Larsen, Copenhagen:

3-Hydroxy-5-(1-aminobutyl)isoxazole

3-Hydroxy-5-(1-aminoethyl)isoxazole

3-Hydroxy-5-(2-aminoethyl)isoxazole

3-Hydroxy-4-(2-aminoethyl)-5-methylisoxazole

3-Hydroxy-4-aminomethyl-5-methylisoxazole

3-Hydroxy-5-aminomethylpyrazole

3-Hydroxy-5-(1-aminopropyl)isoxazole

3-Hydroxy-5-(3-aminopropyl)isoxazole

3-Hydroxy-4-ethyl-5-aminomethylisoxazole

3-Hydroxy-4-methyl-5-(2-aminoethyl)isoxazole

3-Hydroxy-4-methyl-5-aminomethylisoxazole

Nipecotic acid (piperidine-3-carboxylic acid)

Perhydrooxazine-6-carboxylic acid

5,6,7,9,-Tetrahydro-4H-isoxazolo[4,5-d]azepin-3-ol

4,5,6,7-Tetrahydroisoxazolo[4,5-c]pyridin-3-ol

Dr R.H.F. Manske, Waterloo:

Corlumine

Dr A. Meister, New York:

'Cycloglutamic acid'

L-Methionine-SR-sulphoximine phosphate

Dr J.C. Watkins, Bristol:

Ambenoxam

5-Aminoethyltetrazole

1-(2-Aminoethyl)uracil

3-(2-Aminoethyl)uracil

DL-α-(2-Amino-1-methylethyl)benzhydrol

5-Aminomethyltetrazole

1,3-Di-(2-aminoethyl)uracil

4-Fluroglutamic acid diethyl ester

4-Fluroglutamic acid-1-amide

L-Glutamic acid di-tert-butyl ester

N-Methyl-DL-aspartic acid diethyl ester

N-Methyl-DL-aspartic acid di-n-propyl ester

1-(2-Pyridyl)-1,2,4,-triazole-3-sulphonic acid

4-(4-Pyridyl)-1,2,4,-triazole-3-sulphonic acid

4-(3-Pyridyl)-1,2,4-triazole-3-sulphonic acid

Pyridine-2-phosphonic acid

1,2,4-Triazole-3-sulphonic acid

β-(m-Trifluoromethylphenyl)-γ-aminobutyric acid

DL-Willardine

(b) Synthesised in the Department of Pharmacology, John Curtin School of Medical Research, Canberra:

cis-4-Aminocrotonic acid (Mr B. Twitchin)

trans-4-aminocrotonic acid (Dr P.M. Beart and Mr B. Twitchin)

Bicucine methyl ester (Dr P.M. Beart)

Bicuculline methochloride (Dr G.A.R. Johnston)

Bicuculline tetraphenol (Mr B. Twitchin)

Dihydrokainic acid (Dr G.A.R. Johnston)

1-Hydroxy-3-aminopyrrolidone-2 (Dr G.A.R. Johnston)

N-Methyl bicuculleine (Dr G.A.R. Johnston)

N-Methyl-D-aspartic acid (Dr J.C. Watkins and Mr B. Twitchin)

(c) Purchased from commercial suppliers:

Drugs and chemicals not mentioned above were purchased from commercial suppliers.

III. GABA ANALOGUES AS NEURONAL DEPRESSANTS

Despite X-ray analysis and molecular orbital calculations of the GABA molecule and some of its analogues, an investigation of the 'active conformations' of the relatively flexible GABA molecule must still rely heavily upon structure-activity correlation of conformationally restricted GABA analogues. Accordance between such indirectly determined 'active conformations' and low energy conformers of GABA may support the physiological importance of the particular conformational modes (see Section I).

Of the conformationally restricted GABA analogues that have been previously investigated, 4-aminotetrolic acid and muscimol (3-hydroxy-5-aminoethylisoxazole) have GABA-like (see Section I; Curtis, Hösli and Johnston, 1968; Curtis, Duggan, Felix and Johnston, 1971; Curtis, Duggan, Felix, Johnston and McLennan, 1971) postsynaptic effects after microelectrophoretic administration (Beart, Curtis and Johnston, 1971; Johnston, Curtis, de Groat and Duggan, 1968), but differ in their effects on the high affinity uptake of GABA by brain slices. 4-Aminotetrolic acid is a competitive inhibitor of high affinity GABA uptake by rat brain slices (Beart, Johnston and Uhr, 1972), however muscimol is only a weak non-competitive inhibitor of GABA uptake (Johnston, 1971). This rather selective postsynaptic action of muscimol has prompted the biological investigation of a series of compounds structurally related to muscimol as an attempt to evaluate conformationally restricted GABA analogues with specific biological activities. This series of GABA

analogues (see Table 2) has been evaluated as depressants of spinal neurones, and an attempt has been made to correlate structure and activity in order to evaluate the 'active conformers' of GABA. A further aim of this study was to evaluate specific inhibitors of the GABA uptake process in vivo. The inhibition of GABA uptake processes in vivo is of pharmacological interest and may provide information regarding the physiological role of uptake processes.

Trans-4-aminocrotonic acid, like muscimol and 4-aminotetrolic acid is an analogue of GABA in a relatively extended conformation, and both trans-4-aminocrotonic and 4-aminotetrolic acids are competitive inhibitors of the high affinity uptake of GABA by rat brain slices (Beart, Johnston and Uhr, 1972). The actions of muscimol and 4-aminotetrolic acid as GABA agonists with bicuculline-sensitive postsynaptic receptors in the feline spinal cord (Curtis, Duggan, Felix and Johnston, 1971; Beart, Curtis and Johnston, 1971) suggests that extended conformations of GABA are important conformational requirements for interaction of GABA with its postsynaptic receptors. The uptake system, which is competively inhibited by trans-4-aminocrotonic acid and 4-aminotetrolic acids, would appear to have similar conformational requirements. Other studies suggest that folded conformations of GABA may be the active conformations (see Section I), but an analogue of GABA in a folded conformation has yet to be investigated in biological systems. Cis-4-aminocrotonic acid is an analogue of GABA in a folded conformation, and hence its effects on single spinal neurones have been investigated

and compared with those of trans-4-aminocrotonic acid.

A number of aminocyclohexane derivatives were also studied as a series of GABA analogues of restricted conformation. In this series the conformational restriction results from the carbon chain being part of a cyclohexane ring and variation of the distance between the amino and carboxyl functional groups is possible. The potencies of these amino-cyclohexane derivatives as depressants of spinal neurones have been compared with that of GABA.

Although not a GABA analogue of restricted conformation, β-(p-chlorophenyl)-GABA (βCPG) has been included in this study of GABA analogues for several reasons. βCPG (Baclofen, Lioresal) is one of a series of lipophilic derivatives of GABA which more readily penetrates a blood-brain barrier than the parent compound (Faigle and Keberle, 1972a; Faigle and Keberle, 1972b; Keberle and Faigle, 1972), and it has proved of therapeutic benefit in the alleviation of spasticity resulting from a variety of neurological disorders (Birkmayer, 1972; Burke, Andrews and Knowles, 1971). When administered intravenously to cats βCPG reduced mono- and polysynaptic reflexes, and diminished spasticity resulting from either mesencephalic transection or ischaemic decerebration (Bein, 1972). Supraspinal effects appeared to be minimal, and although a mode of action for βCPG similar to that of GABA has been suspected, no clear evidence has yet been obtained to decide this issue (see Bein, 1972). For these reasons βCPG was included amongst the GABA analogues that

were compared with the parent compound in order to determine whether it interacts with bicuculline-sensitive post-synaptic receptors. The information to be thus obtained was sought as a structure-activity relationship and as a possible explanation of the mechanism of action of βCPG when used in the treatment of spasticity.

RESULTS

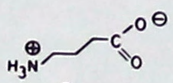
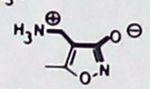
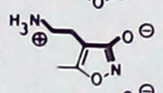
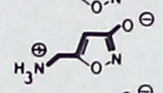
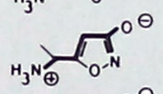
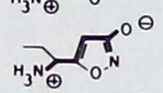
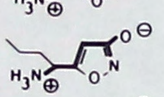
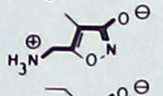
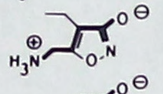
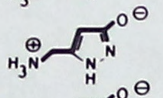
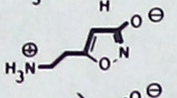
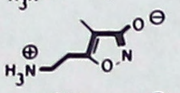
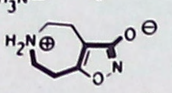
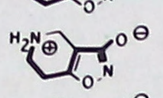
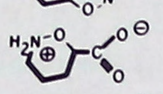
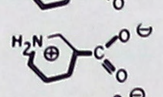
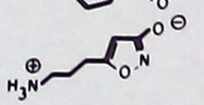
(1) Muscimol derivatives

Twenty-two spinal cord preparations were used to study the effects of the compounds that are contained in Table 2 on Renshaw cells and spinal interneurones, neurones which have a similar sensitivity to GABA and glycine (Curtis, Hösli and Johnston, 1968). Most of these compounds are structurally related to muscimol which has been previously studied (Johnston, Curtis, de Groat and Duggan, 1968; Curtis, Duggan, Felix and Johnston, 1971), and the reported values for muscimol are included in the table.

(a) Depressants of spinal neurones

The approximate potency of the postsynaptic effects of the compounds relative to that of GABA are listed in Table 2. As shown in Fig. 1, 3-hydroxy-5-(1-aminoethyl)-isoxazole (compound IV) an analogue of GABA, clearly inhibited the firing of spinal neurones. The depressant activity of compound IV, which was similar in potency and time course to that of GABA, could be reversibly suppressed by bicuculline methochloride (BMC: see Johnston, Beart, Curtis, Game, McCulloch and MacLachlan, 1972; Section IV), but not by strychnine hydrochloride (2 neurones). As a depressant, compound IV was weaker than muscimol (Johnston, Curtis, de Groat and Duggan, 1968; Curtis, Duggan, Felix and Johnston, 1971). The depressant actions of the structurally related isoxazoles,

TABLE 2 STRUCTURE AND BIOLOGICAL ACTIVITIES OF SOME GABA ANALOGUES OF RESTRICTED CONFORMATION

	COMPOUND	FORMULA	PARENT AMINO ACID	SOLUTION Concentration	SOLUTION pH	No. NEURONES	RELATIVE POTENCY	SENSITIVITY TO BMC	POTENTIATION OF GABA	INHIBITION OF GABA UPTAKE (IC_{50} values$\times 10^6$)	ANTAGONISM OF GLYCINE
	GABA			0.2 M	3.5(HCl)		---	+			
I	3-Hydroxy-4-aminomethyl-5-methylisoxazole		β-Alanine	0.2 M	TsOH	4	0		0		0
II	3-Hydroxy-4-(2-aminoethyl)-5-methylisoxazole		GABA	0.2 M	HBr	4	0		0		0
III	3-Hydroxy-5-aminomethylisoxazole (muscimol)		GABA				----	+		>1000	
IV	3-Hydroxy-5-(1-aminoethyl)isoxazole (H1AI)		GABA	0.2 M	HBr	21	---	+		>1000	
V	3-Hydroxy-5-(1-aminopropyl)isoxazole		GABA	0.2 M	3.5(HCl)	11	--	0	0		0
VI	3-Hydroxy-5-(1-aminobutyl)isoxazole		GABA	0.2 M	3.5(HCl)	13	--	0	0		0
VII	3-Hydroxy-4-methyl-5-aminomethylisoxazole		GABA	0.2 M	HBr	9	-	n.t.	0		0
VIII	3-Hydroxy-4-ethyl-5-aminomethylisoxazole		GABA	0.2 M	HBr	5	0		0		0
IX	3-Hydroxy-5-aminomethylpyrazole ("azamuscimol")		GABA	0.2 M	2 HCl	6	--	n.t.	0		0
X	3-Hydroxy-5-(2-aminoethyl)isoxazole (H2AI)		δ-Aminovaleric acid	0.2 M	3.5(HCl)	7	---	+			
XI	3-Hydroxy-4-methyl-5-(2-aminoethyl)isoxazole		δ-Aminovaleric acid	0.2 M	HBr	10	0		0		0
XII	5,6,7,8-Tetrahydro-4H-isoxazolo[4,5-d]azepin-3-ol		δ-Aminovaleric acid	0.2 M	HBr	4	0		0		+
XIII	4,5,6,7-Tetrahydroisoxazolo-[4,5-c]pyridin-3-ol		δ-Aminovaleric acid	0.2 M	HBr	15	(-)	n.t.	+	624	+
XIV	Perhydro-1,2-oxazine-6-carboxylic acid		δ-Aminovaleric acid	1.0 M	2.5(HCl)	14	(-)	n.t.	(+)	380	0
XV	Piperidine-3-carboxylic acid (nipecotic acid)		δ-Aminovaleric acid	0.2 M	3.5(HCl)	20	(-)	n.t.	+	9	+
XVI	3-Hydroxy-5-(3-aminopropyl)-isoxazole		ε-Aminocaproic acid	0.2 M	HBr	20	(-)	n.t.	0		0

(See the following page for notes to this table).

NOTES TO TABLE 1: STRUCTURE AND BIOLOGICAL ACTIVITIES OF SOME ANALOGUES OF RESTRICTED CONFORMATION

1. The structural similarities of the compounds to the parent amino acids β-alanine, GABA, δ-aminovaleric acid and ε-aminocaproic acid are indicated by heavy black lines.

2. The properties of the solution are those of the solution used in the microelectrophoretic experiments. The following abbreviations have been used:

   HBr, hydrobromide salt of the compound
   HCl, hydrochloride salt of the compound
   (HCl), hydrochloric acid used to adjust pH of the solution
   TsOH, toluene sulphonate salt of the compound.

   Compounds containing asymetric centres were racemates.

3. The potencies of the compounds as depressants are cited relative to that of GABA (---).

4. The IC_{50} values (μM) are for inhibition of GABA uptake by rat brain slices, the values for muscimol being that of Johnston (1971) and the remaining values are from Krogsgaard-Larsen and Johnston (1975).

5. Antagonism of the depressant action of glycine: O, no activity; +, significant activity.

6. Effects of doubtful significance are cited in brackets.

7. Abbreviations:
   BMC, bicuculline methochloride
   IC_{50}, concentration of compound (μM) causing 50% inhibition of the uptake of [^{3}H] GABA by rat brain slices
   n.t., not tested.

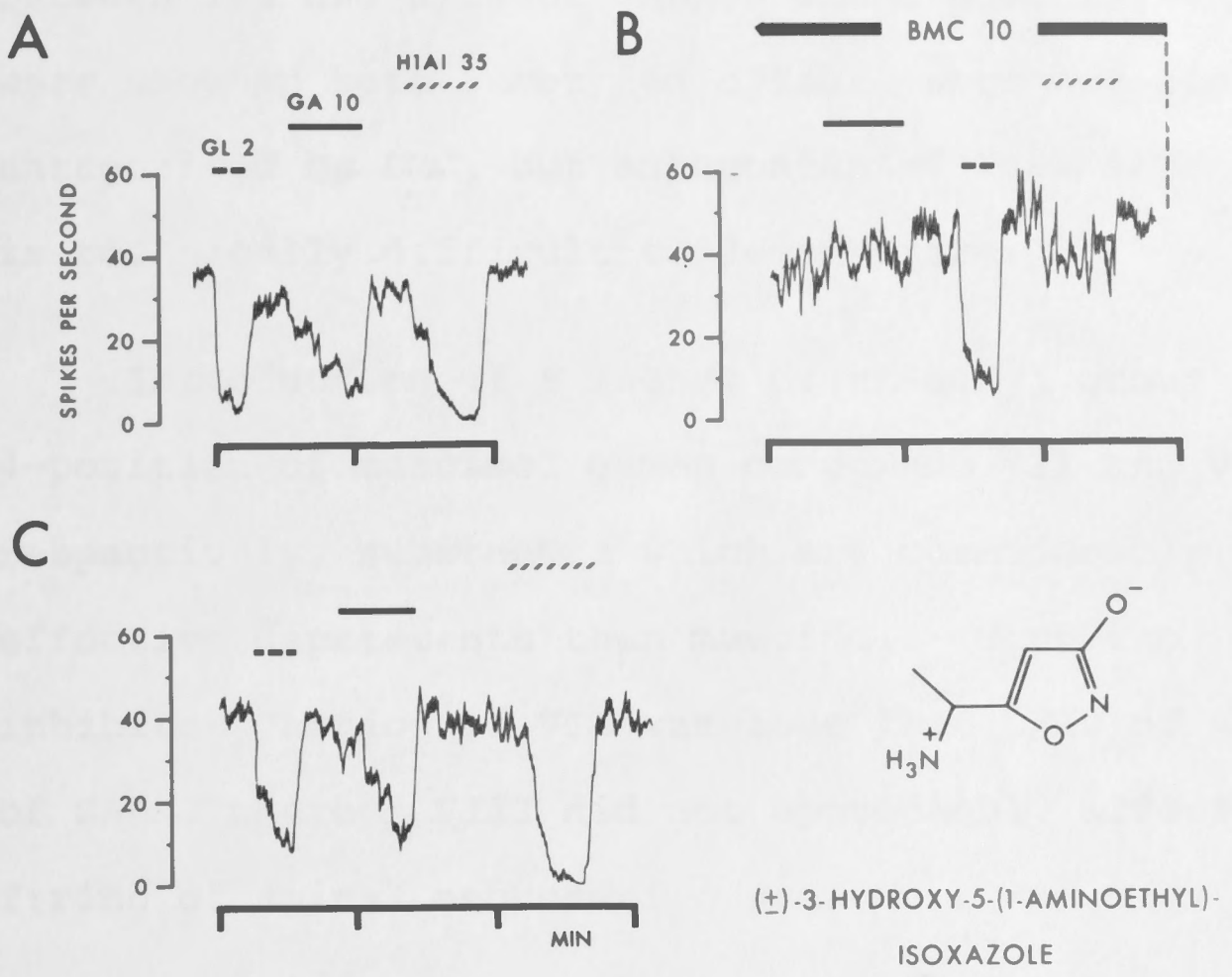


Fig. 1:

The effect of bicuculline methochloride (BMC) on the inhibition of firing of a spinal interneurone by GABA, glycine and 3-hydroxy-5-(1-aminoethyl)isoxazole (H1AI). The rate of firing of the interneurone was maintained by the continuous electrophoretic ejection of DL-homocysteate (DLH), the rate of ejection of which was varied to allow for the excitant effect of BMC. GABA (GA, 10 nA), glycine (GL, 2 nA) and 3-hydroxy-5-(1-aminoethyl)isoxazole (H1AI, 35 nA) were administered for the periods indicated by the respective solid and broken horizontal bars. The solid horizontal bar and vertical broken line indicates the period of continuous ejection of BMC from a solution of 10 mM in 165 mM NaCl. A, control responses during the administration of DLH, 12 nA; B, responses during the administration of BMC with a current of 10 nA, DLH, 8 nA; C, 2.5 minutes after the termination of the BMC ejection. Ordinates: firing frequency in spikes per second. Abscissae: time in minutes.

compounds V and VI, were considerably weaker, being between 1/5 and 1/10 of GABA. These effects, which were slow in both onset and offset, were not clearly antagonised by BMC, but antagonism of weak depressants is technically difficult to demonstrate.

Introduction of a methyl or an ethyl group in the 4-position of muscimol gives compounds VII and VIII, respectively, substances which are considerably less effective depressants than muscimol. Thus the inhibitory action of VII was less than 1/10 of that of GABA, whereas VIII did not appreciably affect the firing of spinal neurones.

3-Hydroxy-5-(2-aminoethyl)isoxazole (compound X), an analogue of δ-aminovaleric acid, was a potent depressant of spinal neurones, comparable in potency to GABA. The inhibitory effect was rather slow in onset and was reversibly antagonised by BMC as shown in Fig. 2. Analogous to the 4-alkyl substituted muscimol derivatives VII and VIII, the 4-methyl derivative (compound XI) of 3-hydroxy-5-(2-aminoethyl) isoxazole was less than 1/10 as potent as GABA.

3-Hydroxy-5-aminomethylpyrazole ('azamuscimol') is structurally related to muscimol, but the physico-chemical properties of the pyrazole nucleus are quite different from those of the isoxazole moiety of muscimol. 'Azamuscimol' was a depressant of the firing of spinal neurones, being approximately 1/3 as active as GABA, but some variation in the sensitivity of different neurones was observed.

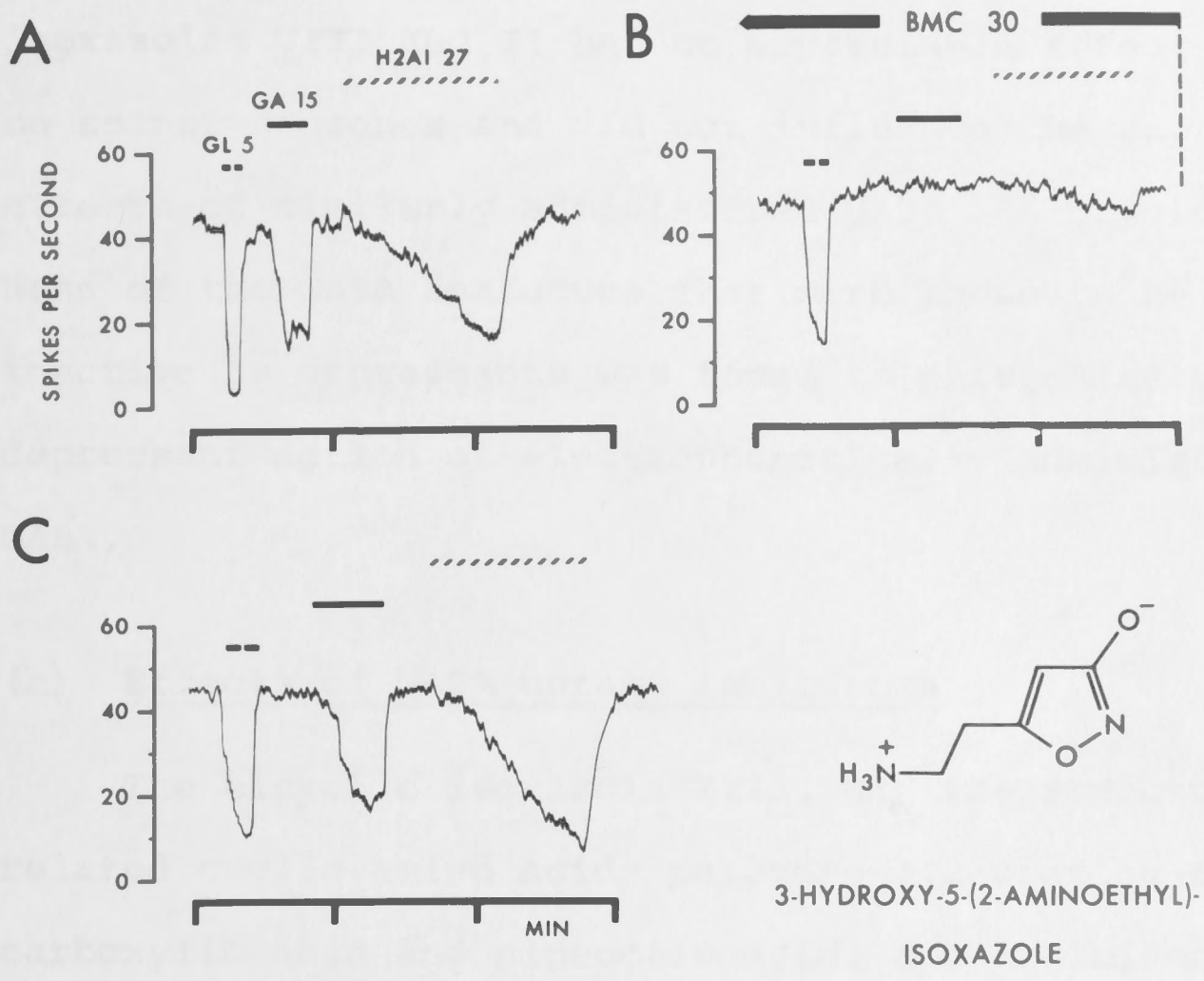


Fig. 2:

The effect of bicuculline methochloride (BMC) on the inhibition of firing of a spinal interneurone by glycine, GABA and 3-hydroxy-5-(2-aminoethyl)isoxazole (H2AI). The rate of firing of the interneurone was maintained by the continuous electrophoretic ejection of DL-homocysteate (DLH, 5 nA). Glycine (GL, 5 nA), GABA (GA, 15 nA) and 3-hydroxy-5-(2-aminoethyl)isoxazole (H2AI, 27 nA) were administered for the periods indicated by the respective solid and broken horizontal lines. The solid bar and the vertical broken line indicates the period of continuous ejection of BMC from a solution of 10 mM in 165 mM NaCl. A, responses before; B, during the administration of BMC with a current of 30 nA; C, 1 minute after the termination of the BMC ejection.

3-Hydroxy-5-(3-aminopropyl)isoxazole (compound XVI) an analogue of ε-aminocaproic acid, was a weak depressant of the firing rate of spinal neurones. The isoxazoles VIII and II had no appreciable effects on spinal neurones and did not influence the depressant effects of similarly administered GABA and glycine. None of the GABA analogues that were shown to be inactive as depressants was found to antagonise the depressant action of electrophoretically administered GABA.

(b) Effects of GABA uptake inhibitors

The bicyclic isoxazole XIII, and the structurally related cyclic amino acids perhydro-1,2-oxazine-6-carboxylic acid and nipecotic acid, are inhibitors of the high affinity uptake of GABA by rat brain slices (Krogsgaard-Larsen and Johnston, 1975). The remaining compounds in Table 2 did not inhibit GABA uptake, in vitro, except muscimol (Johnston, 1971) and compound IV, both of which are very weak inhibitors of GABA uptake (Krogsgaard-Larsen and Johnston, 1975). These findings prompted investigation of the effect on single neurones of XIV (perhydro-1,2-oxazine-6-carboxylic acid), nipecotic acid and the structurally related bicyclic isoxazoles XII and XIII. Fig. 3 illustrates the enhancement of the depressant action of electrophoretically administered GABA by similarly administered nipecotic acid.

Nipecotic acid was a very weak depressant of the firing of spinal neurones, an effect which was very

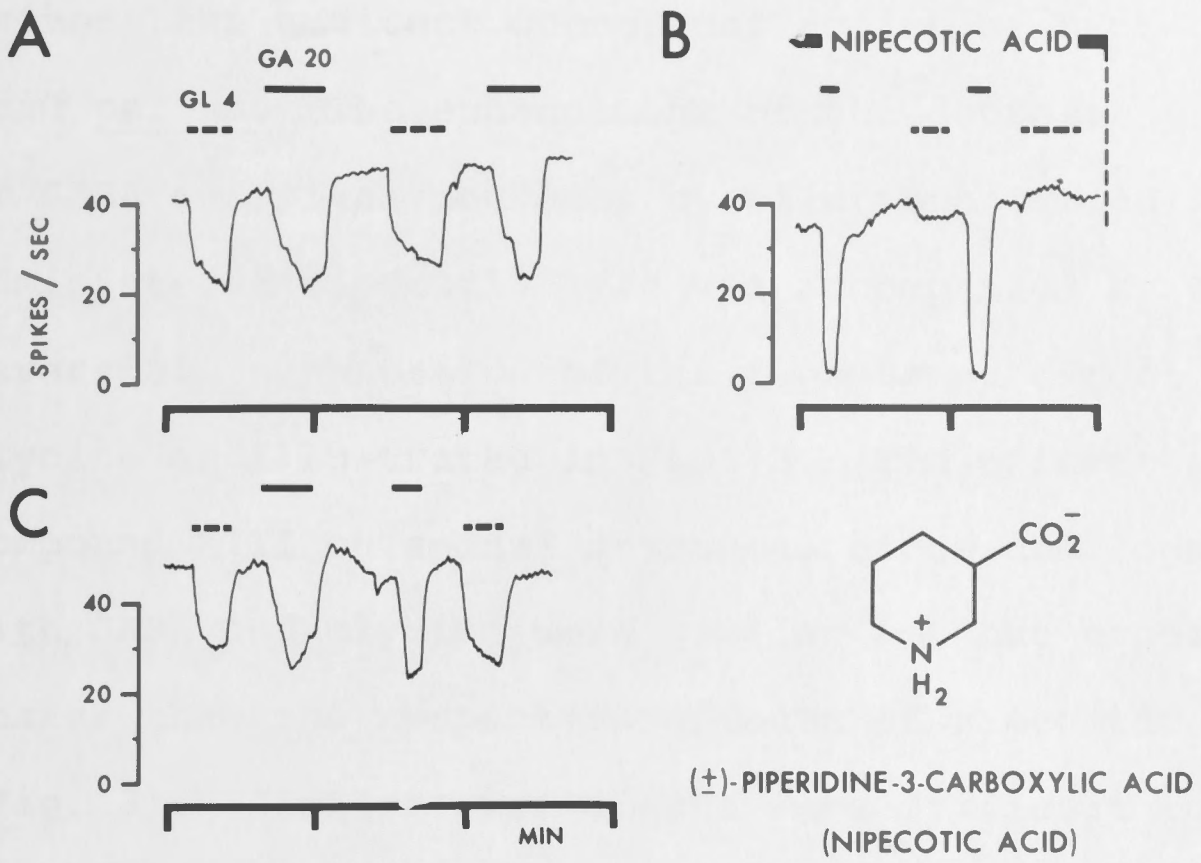


Fig. 3:

The effect of nipecotic acid on the inhibition of firing of a spinal interneurone by glycine and GABA. The rate of firing of the interneurone was maintained by the continuous electrophoretic ejection of DL-homocysteate (DLH). Glycine (GL, 4 nA) and GABA (GA, 20 nA) were administered for the periods indicated by the broken and solid horisontal lines, respectively. Nipecotic acid was administered for the period indicated by the solid horizontal bar and broken vertical line. A, control responses during the administration of DLH, 6 nA; B, responses during the administration of DLH (12 nA) and nipecotic acid with a current of 30 nA which was subsequent to a current of 20 nA for 2 minutes; C, responses 1.5 minutes after termination of the nipecotic acid ejection and during the administration of DLH (7 nA).

slow in both onset and offset. This effect may have been a consequence of the accumulation of synaptically released GABA following the inhibition of its uptake rather than a direct depressant action of nipecotic acid *per se*. The enhancement of the depressant effect of GABA on spinal neurones by electrophoretically administered nipecotic acid was accompanied by a reversible suppression of the depressant effect of glycine as illustrated in Fig. 3. The effects of compound XIII on spinal neurones, alone and together with GABA and glycine were similar to, but probably weaker than the respective effects of nipecotic acid (Fig. 3). Similar experiments were difficult to perform with perhydro-1,2-oxazine-6-carboxylic acid because of the relatively low conductance of acidified aqueous solutions, but selective enhancement of the depressant activity of GABA by electrophoretically administered perhydro-1,2-oxazine-6-carboxylic acid was demonstrated on one neurone. The isoxaxole XII did not significantly influence the action of GABA under similar conditions.

(c) Glycine antagonists

A reversible antagonism of the depressant action of glycine similar to that of nipecotic acid as illustrated in Fig. 3 was demonstrated after micro-electrophoretic administration of bicyclic isoxazoles XII and XIII. The isoxazole XII did not influence the firing rate of spinal interneurones and it does not influence the uptake of either GABA or glycine by rat brain slices (Krogsgaard-Larsen and Johnston, 1975). This isoxazole thus appears to be a rather specific

antagonist of the postsynaptic action of glycine, although weak when compared with strychnine (Curtis, Duggan and Johnston, 1971).

(2) Cis and trans-4-aminocrotonic acids as depressants of spinal neurones

Cis-4-aminocrotonic acid depressed the firing of all 6 interneurones (2 preparations) tested and was approximately 1/4 as potent as GABA. This depressant action could not be antagonised by either BMC (3 neurones) or strychnine hydrochloride (2 neurones) in concentrations adequate to block the effects of GABA or glycine, respectively. In contrast, trans-4-aminocrotonic acid, tested on 20 neurones (6 preparations) was approximately as potent as GABA as a depressant and this action could be blocked by BMC (6 neurones), but not by strychnine (3 neurones). The firing frequency versus time recordings and filmed records indicated that the time courses of onset and recovery for trans-4-aminocrotonic acid were similar to those for GABA.

Fig. 4A-C is a recording from a spinal interneurone that was being excited by the continuous administration of DLH. This neurone responded in a similar manner to GABA (10 nA), trans-4-aminocrotonic acid (20 nA) and cis-4-aminocrotonic acid (50 nA). 10 nA of BMC reduced the depressant effects of GABA and trans-4-aminocrotonic acid but not that of cis-4-aminocrotonic acid. Strychnine hydrochloride (30 nA) had no significant effect on any of the three depressants.

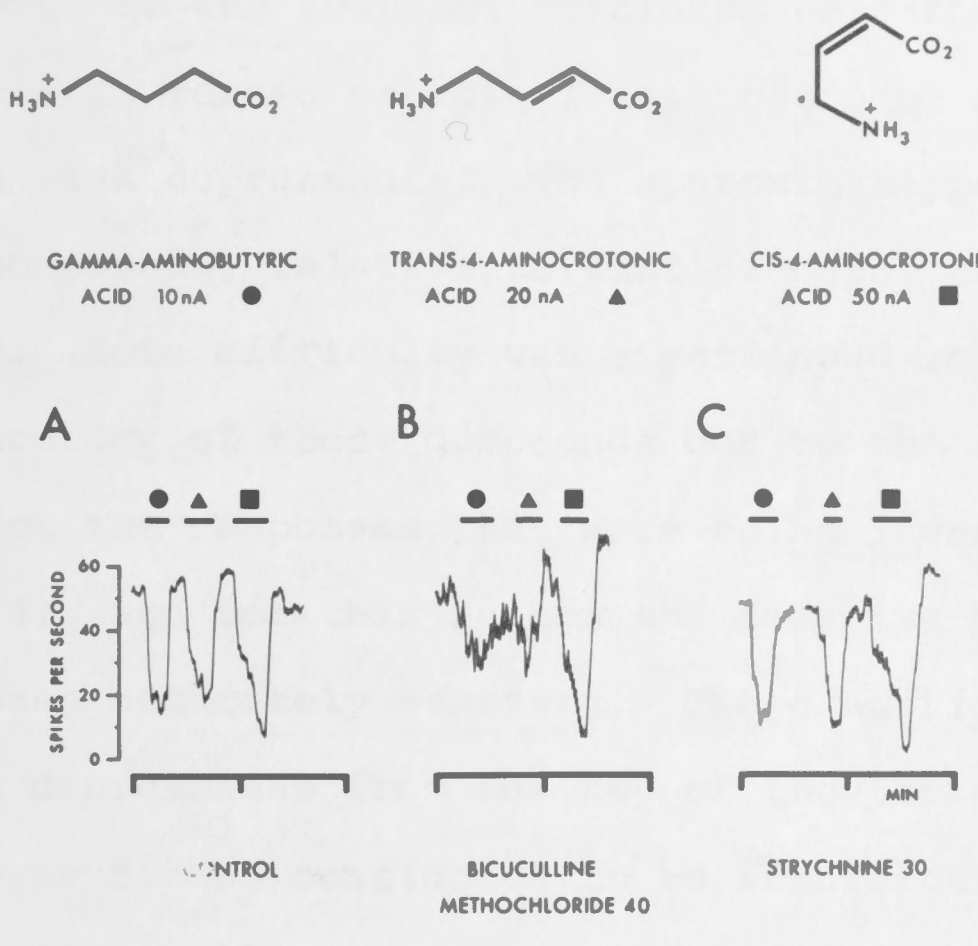


Fig. 4:

Structures of GABA, trans- and cis-4-aminocrotonic acids and their depressant effects on the firing of a spinal interneurone excited by the continuous electrophoretic ejection of DL-homocysteate (DLH, 20 nA). The depressant amino acids were administered for the times indicated by the horizontal black bars and symbols. A, control observations; B, during the administration of bicuculline methochloride (BMC, 10 mM in 165 mM NaCl) with a current of 40 nA for 3 minutes; C, after recovery from the effects of BMC and during the administration of strychnine hydrochloride (2 mM in 165 mM NaCl) with a current of 30 nA for 3 minutes. Maximum effects of BMC and strychnine are illustrated.

(3) Cyclohexane derivatives as depressants of spinal neurones

Three of the four aminocyclohexane carboxylic acids that were tested on spinal interneurones and Renshaw cells were weak depressants. The approximate potencies of these compounds, relative to that of GABA, are shown in Table 3. Some difficulty was experienced in studying the pharmacology of these compounds due to the small magnitude of the responses that were being investigated (see Fig. 5), and for this reason the relative potencies have not been accurately assessed. The gradation of potency as depressants from the top of the table to the bottom, however, was considered to be significant. Thus the four aminocyclohexane carboxylic acids were weaker than GABA, cis-3-aminocyclohexane-1-carboxylic acid was the most potent and cis-2-aminocyclohexane-1-carboxylic acid was inactive. Fig. 5 illustrates a comparison of the potencies of cis-2- and trans-2-aminocyclohexane-1-carboxylic acids. In this comparison, on a Renshaw cell excited by DLH (12 nA), the trans-2- derivative was approximately one-fifth as potent as GABA, but the cis-2-derivative was much weaker or inactive and it was impossible to distinguish between a true depression and that due to current alone, for the same current of strychnine hydrochloride produced a depression that was similar to that due to current passed through the cis-2- barrel.

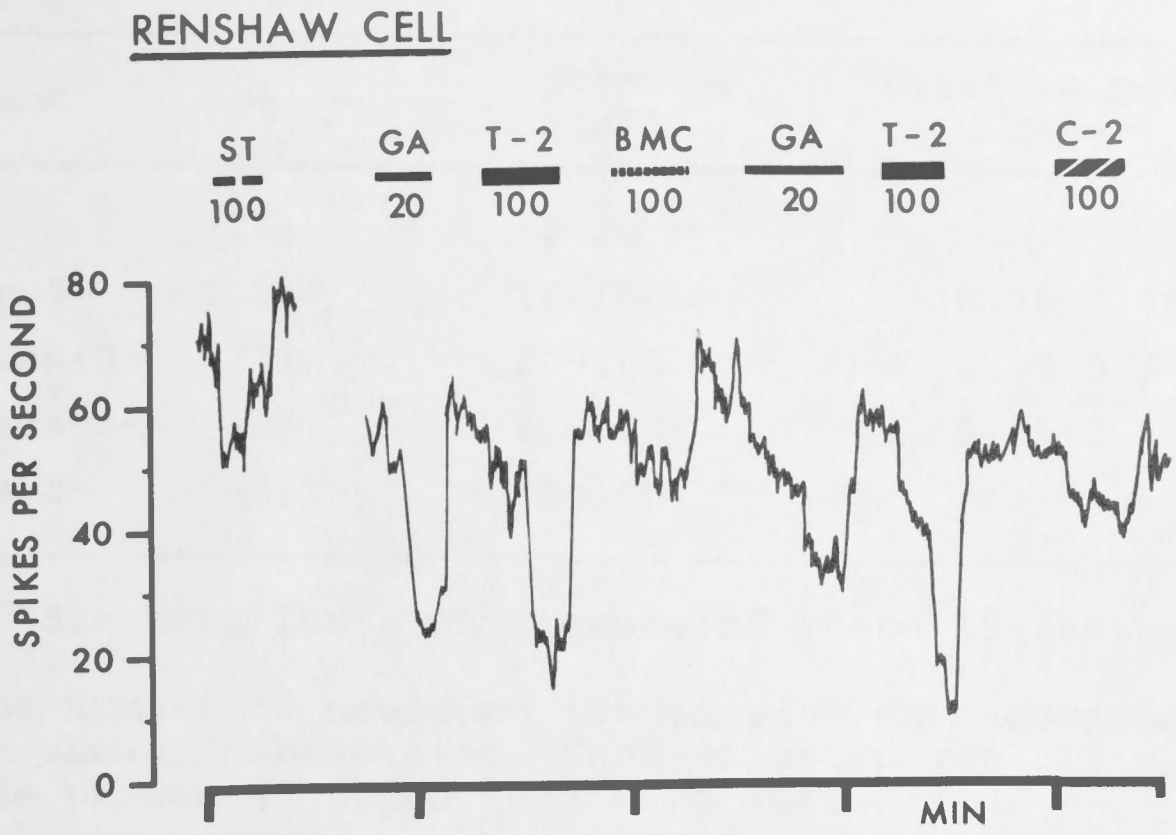


Fig. 5:

The effects of GABA, cis-2- and trans-2-aminocyclohexane-1-carboxylic acids on the firing of a Renshaw cell which was excited by the continuous electrophoretic administration of DL-homocysteate (DLH, 12 nA). Cis-2-aminocyclohexane-1-carboxylic acid and strychnine hydrochloride (2 mM in 165 mM NaCl) were retained by the same anionic currents and thus their ejection with currents of 60 nA represented equal current change. The periods of ejection of cis-2-aminocyclohexane-1-carboxylic acid (cis-2-, 60 nA), trans-2-aminocyclohexane-1-carboxylic acid (trans-2-, 60 nA), strychnine hydrochloride (strych, 60 nA) and GABA (GA, 12 nA) are indicated by the respective solid and broken horizontal lines.

TABLE 3 Relative potencies of aminocyclohexane-1-carboxylic acids as depressants of spinal neurones

Compound		Current (nA)	Relative potency range
GABA	(27)	0-20	1
(±)-cis-3-	(12)*	10-100	0.20-0.25
(±)-trans-3-	(23)	20-100	0.05-0.5
(±)-trans-2-	(9)	30-100	0.05-0.2
(±)-cis-2-	(5)	30-100	-

* See text for significance of these 12 tests.

The number of neurones tested with each compound is shown in brackets. Each solution was 0.2 M and the pH was adjusted to 3 with HCl.

Cis-3-aminocyclohexane-1-carboxylic acid was unusual in that it clearly depressed 2 of the 12 neurones on which it was tested, 6 of the remaining 10 neurones were weakly excited by this compound, but depressed by GABA. As a depressant, cis-3-aminocyclohexane-1-carboxylic acid was clearly more potent than trans-3-aminocyclohexane-1-carboxylic acid when the two were directly compared as depressants on 2 neurones (the only 2 that were depressed by the cis-3- derivative). Cis-2-aminocyclohexane-1-carboxylic acid was very weak or inactive when tested on 5 neurones, a finding that was consistent with the unpublished results of D.R. Curtis.

The relatively low potencies of the three aminocyclohexane-1-carboxylic acids that were weak depressants precluded an accurate determination of their sensitivities to BMC and strychnine hydrochloride. Each depressant compound was tested on a minimum of two neurones during

the continuous administration of either strychnine hydrochloride or BMC and although the depressant actions appeared to be insensitive to these antagonists uniquivocal results were not obtained.

(4) β-(p-Chlorophenyl)-GABA (βCPG) as a depressant of neuronal activity

(a) Spinal interneurones

When administered with cationic currents (10-80 nA) βCPG reversibly reduced the firing rates of spontaneously active or chemically excited spinal interneurones (36 neurones, 16 preparations). The onset of the effect was slow, the potency of βCPG was approximately half that of GABA (24 equipotent current ratio determinations) and in contrast to the rapid recovery of firing which invariably occurs after the termination of GABA-ejecting electrophoretic currents, recovery after βCPG was prolonged, frequently taking 1-2 minutes (see Fig. 6). The tracings of Fig. 6A plot the firing of a spontaneously active dorsal horn interneurone, and compare the effectiveness and time course of depression produced by glycine, GABA and βCPG. Similar results were obtained with neurones fired by L-glutamate, L-aspartate or DLH. As illustrated in Fig. 6B, concentrations of BMC adequate to totally block the effect of GABA had little or no action on the depression of firing by βCPG (3 spinal interneurones). Furthermore, concentrations of strychnine hydrochloride sufficient to abolish the effect of glycine (Fig. 6C) did not modify the action of βCPG (3 spinal interneurones). Thus neither GABA nor glycine receptors appeared to be involved in the depressant

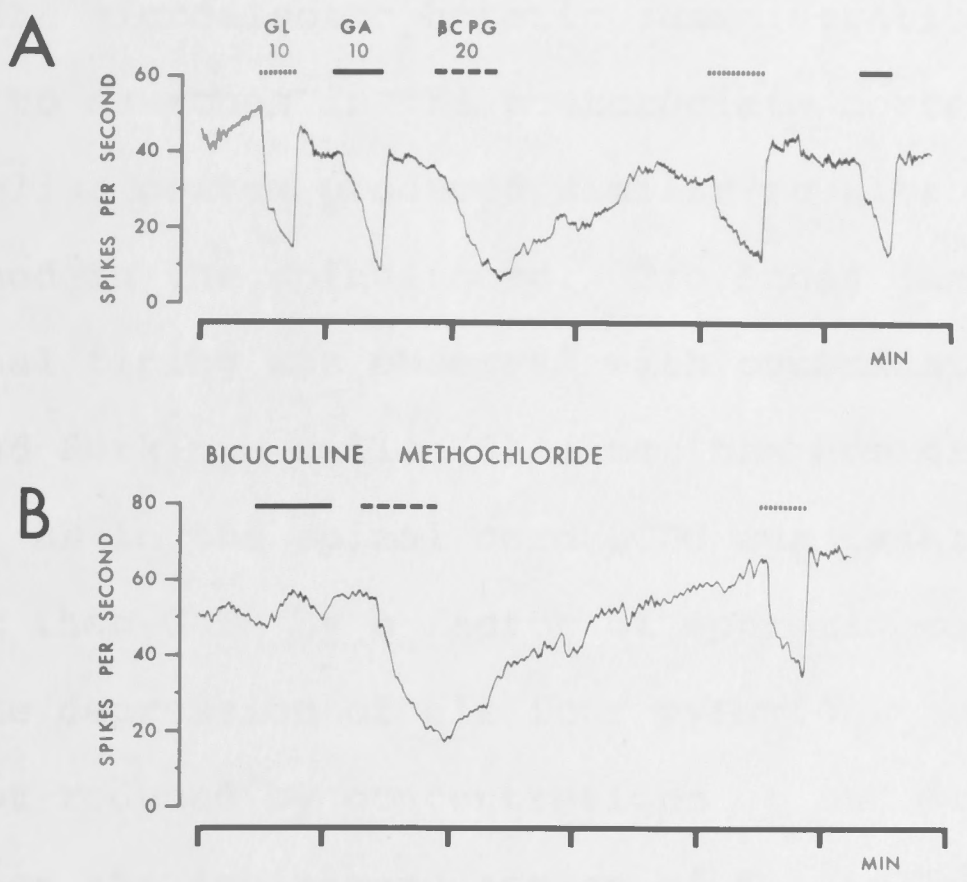


Fig. 6:

The effects of electrophoretically administered glycine, GABA and βCPG, indicated by dotted, solid and broken horizontal lines, on the spontaneous firing of a spinal interneurone. A, control observations of glycine (10 nA), GABA (10 nA) and βCPG (20 nA); B, during the microelectrophoretic administration of bicuculline methochloride (BMC, 10 mM in 165 mM NaCl), 15 nA for 1.4 min, 30 nA for 0.4 min and throughout the period of the illustrated recording; C, after recovery from BMC and during the administration of strychnine (2 mM in 165 mM NaCl) with a current of 20 nA.

action of βCPG.

(b) Pyramidal tract neurones and Purkinje cells

The microelectrophoretic administration of βCPG close to neurones in the postcruciate cortex and vermal cerebellar cortex produced similar results to those obtained in the spinal cord. Prolonged depression of neuronal firing was observed with pyramidal tract cells (5) and Purkinje cells (6) after the administration of βCPG. As in the spinal cord βCPG was usually less potent than GABA by a factor of approximately 0.5, and the depression of all four pyramidal tract neurones was not reduced by concentrations of BMC adequate to suppress the inhibitory action of initially approximately equi-effective amounts of GABA (Fig. 7).

(c) Renshaw cells

Additional evidence suggesting that neither GABA nor glycine receptors were involved in the depressant action of βCPG was obtained from observations made on 21 Renshaw cells, neurones having a sensitivity to glycine and GABA of the same order of magnitude as dorsal horn interneurones (Curtis, Hosli and Johnston, 1968). As illustrated in Fig. 8A and B, βCPG was a weak depressant of the firing of Renshaw cells when compared with glycine and GABA. The micropipette used for both neurones was that used to study the inter-neurones of Fig. 6. In many cases difficulty was experienced in determining whether the effects observed on Renshaw cells were not due merely to the electro-phoretic current, and with all cells the depression was of comparable duration to that of GABA and glycine,

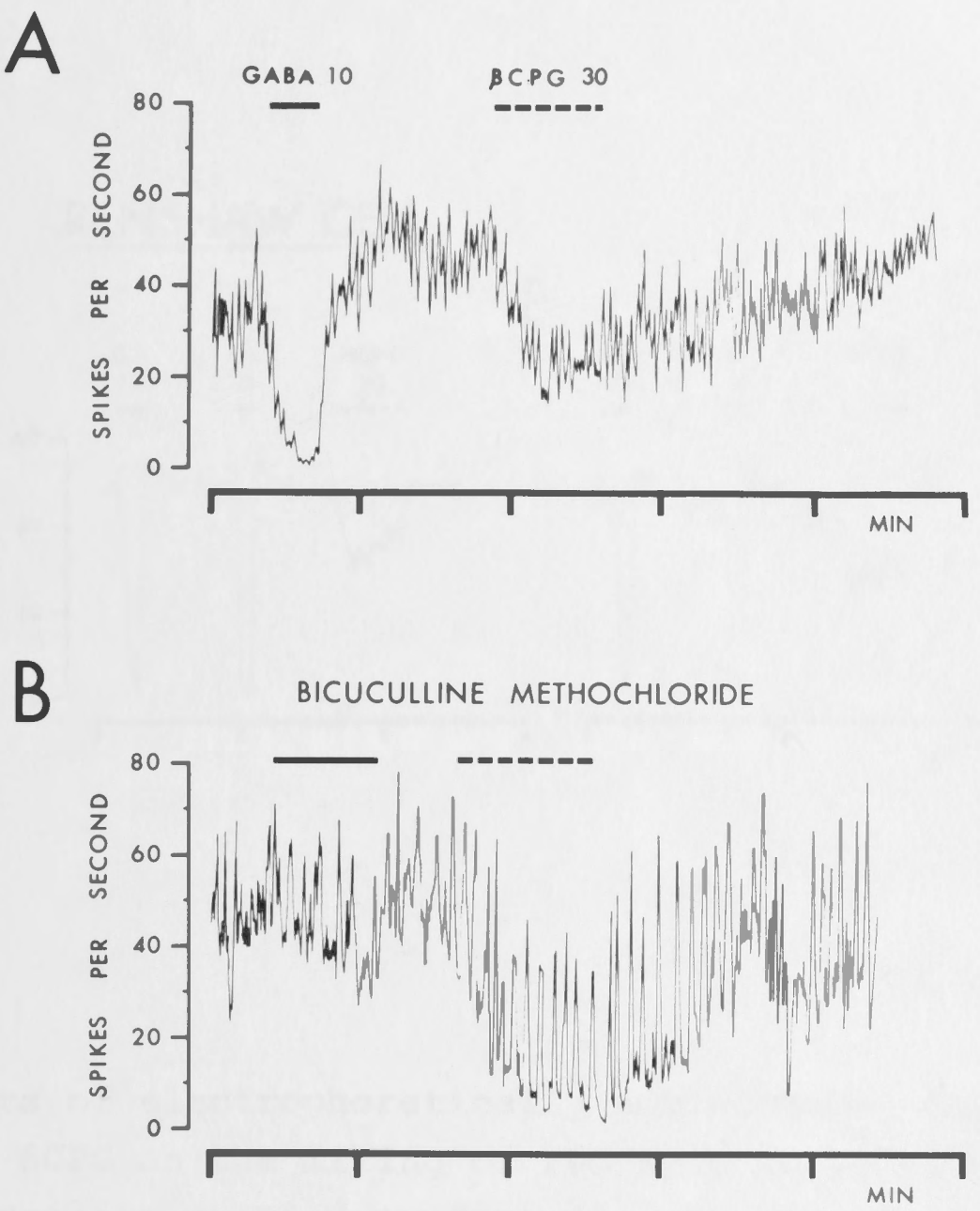


Fig. 7:

Effect of GABA and βCPG on the firing of a pyramidal tract neurone of the postcruciate cortex, maintained with DL-homocysteate (DLH). A, GABA (10 nA), βCPG (30 nA) and DLH (12 nA, continuous); B, 1 min after beginning the ejection of bicuculline methochloride (BMC, 10 mM in 165 mM NaCl) with a current of 40 nA. The increased firing rate was diminished by reducing the DLH current to 7.5 nA, but the record shows periodic bursts of high frequency firing.

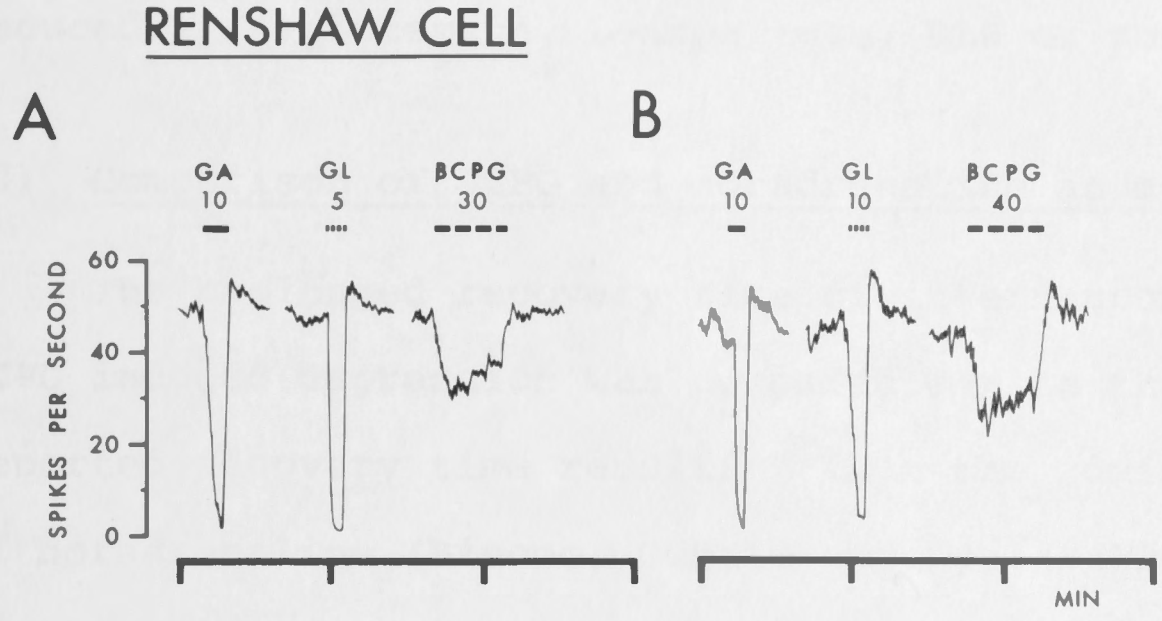


Fig. 8:

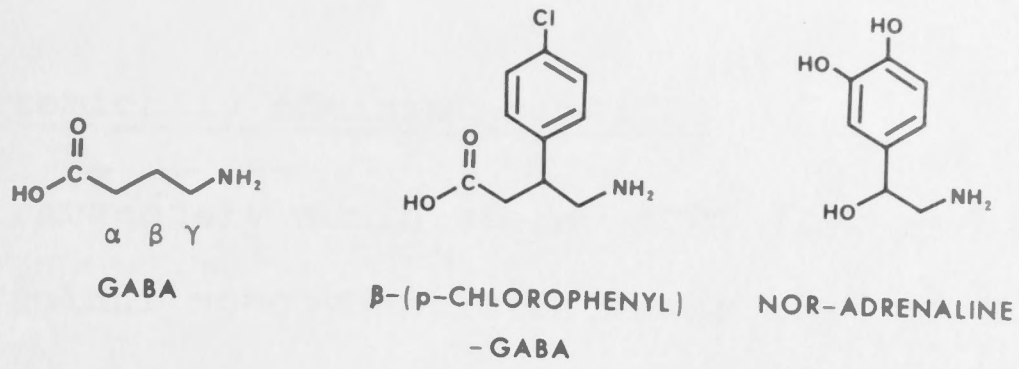
Effects of electrophoretically administered GABA, glycine and βCPG on the firing of two Renshaw cells maintained by DL-homocysteate (DLH). A, GABA (10 nA), glycine (5 nA), βCPG (30 nA) and DLH (12.5 nA, continuous); B, GABA (10 nA), glycine (10 nA), βCPG (40 nA) and DLH (9 nA, continuous).

prolonged depression never being observed. Electrophoretic currents of 30-50 nA of βCPG had no effect on the submaximal ventral root response of 4 Renshaw cells, although GABA (10-20 nA) reduced the response to about 50% of control. No differences were observed in the sensitivity to βCPG of Renshaw cell firing induced by L-glutamate, L-aspartate, DLH or acetylcholine.

(d) Comparison of βCPG and noradrenaline as depressants

The prolonged recovery time of interneurones from βCPG induced depression was comparable with the reported recovery time resulting from the administration of noradrenaline (Biscoe, Curtis and Ryall, 1966). Of the 15 interneurones tested with both βCPG and noradrenaline (20-80 nA), however, only 5 responded to both depressants with a slow rate of recovery. Four of these 15 interneurones responded slowly to βCPG and not to noradrenaline, and the remaining 6 responded to βCPG with the usual slow rate of recovery, but the response to noradrenaline was of a faster time course (Fig. 9A). The slow onset of the first response to GABA (10 nA) in Fig. 9A reflects the lengthy period, prior to this ejection, during which no electrophoretic ejection currents had been passed through the GABA barrel.

The responses of 10 Renshaw cells to noradrenaline were also variable in time course, being either slow (Fig. 9B) (2 cells), moderate (2 cells) or fast (3 cells) in their rates of recovery, but cells which were apparently not affected by βCPG (10-80 nA) were



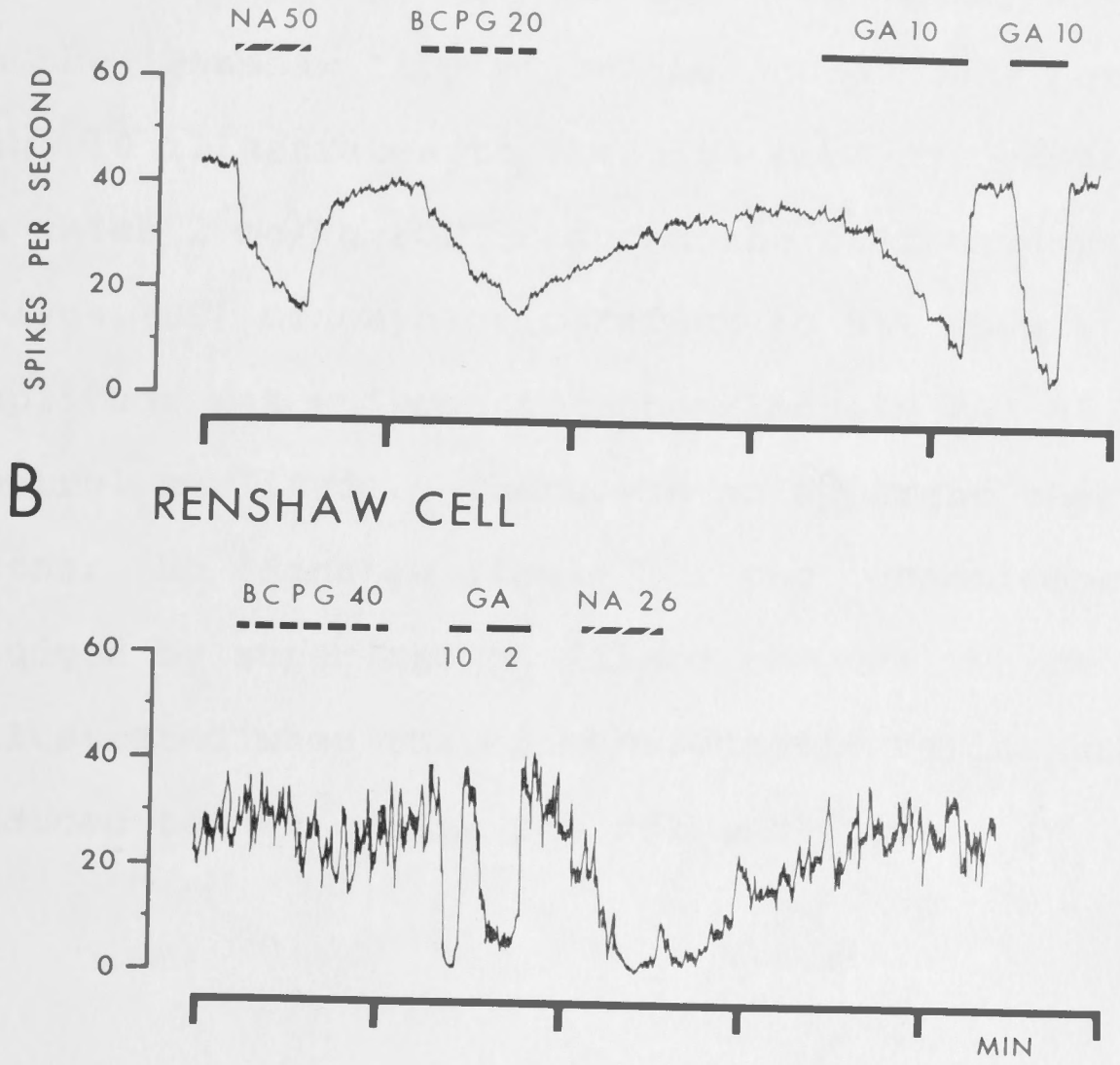


Fig. 9:

The structures and effects of electrophoretically administered βCPG, noradrenaline and GABA on a spinal interneurone and a Renshaw cell. The rates of firing of the interneurone and Renshaw cell were maintained by the continuous electrophoretic administration of DL-homocysteate (DLH) and acetylcholine (ACh) respectively, and the periods of administration of βCPG, GABA (GA) and noradrenaline (NA) are indicated by the respective solid and broken horizontal lines. A, the effects of NA (50 nA), βCPG (20 nA), GABA (10 nA) and DLH (12 nA, continuous) on a spinal interneurone; B, the effects of βCPG (40 nA), GABA (10, 2 nA), NA (26 nA) and ACh (4 nA, continuous) on a Renshaw cell.

readily depressed by noradrenaline (20-80 nA).

(e) Systemically administered βCPG

Intravenously administered βCPG (1-2 mg/kg) reduced spinal monosynaptic reflexes with a simultaneous reduction of dorsal root potentials (DRPs) (4 preparations). There was, however, no apparent change in the 'Renshaw ripple' or the 'quadriceps notch'. Fig. 10 illustrates the results from one experiment in which 2 mg/kg βCPG reduced the gastrocnemius-soleus (GS) monosynaptic reflex to 55% when the DRP amplitude was reduced to approximately 70% of its control amplitude. There was no apparent change in either the 'Renshaw ripple' or the 'quadriceps notch' (judged by superimposed filmed records) which are illustrated when the GS monosynaptic reflex had been reduced to 30% of its control amplitude.

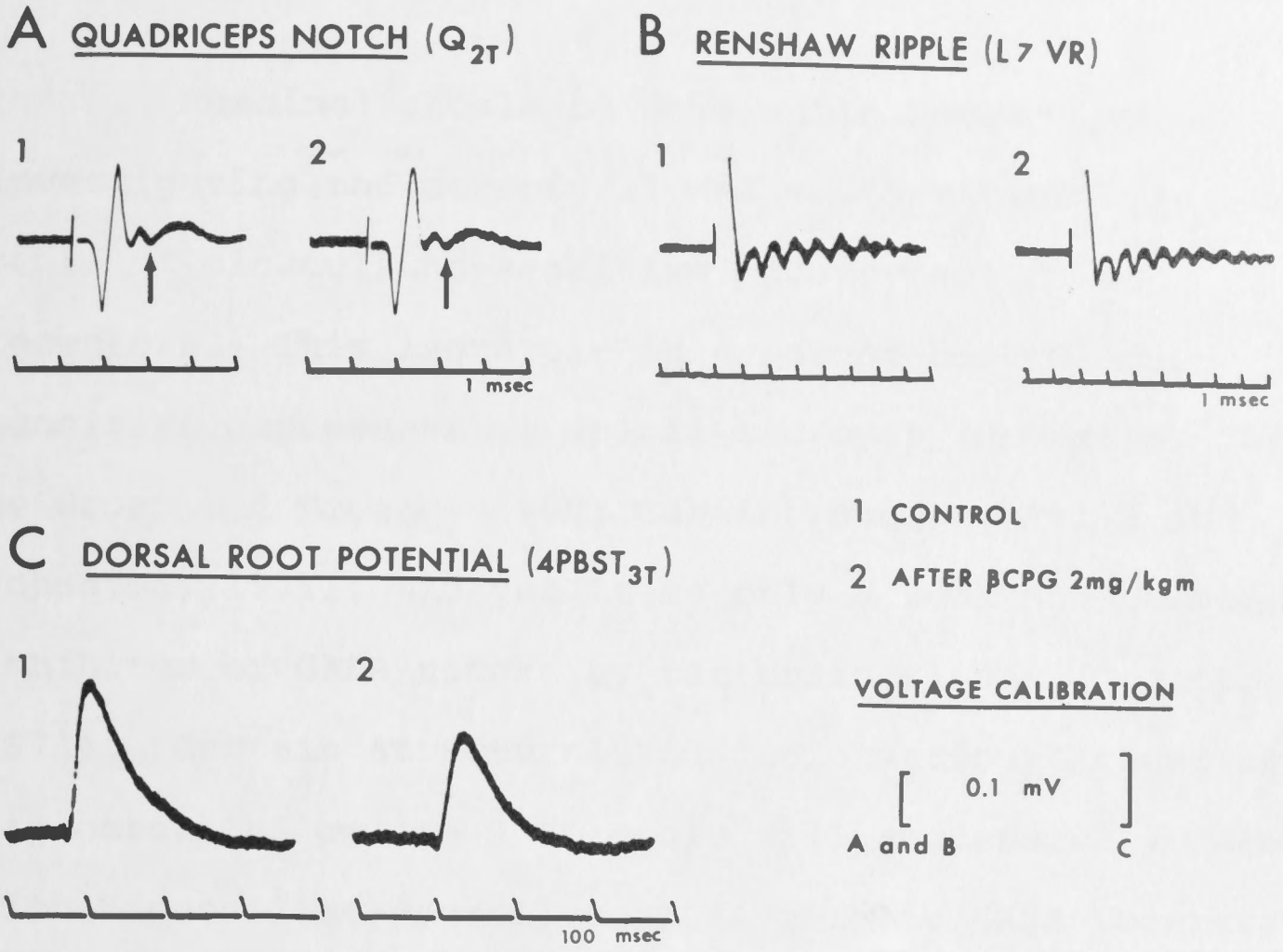


Fig. 10:

The effects of β-(p-chlorophenyl)-GABA (βCPG) on the 'quadriceps notch', 'Renshaw ripple' and dorsal root potential. A, the 'quadriceps notch' (indicated by the arrow) recorded from the dorsal surface of the spinal cord in response to stimulation of the quadriceps nerve at 2 x threshold (Q_{2T}); B, the 'Renshaw ripple' recorded from the dorsal surface of the spinal cord in response to stimulation of the seventh lumbar ventral root (L7 VR); C, the dorsal root potential (DRP) recorded from the most caudal rootlet of the sixth lumbar dorsal root (amplifier time constant 1 sec) in response to stimulation of the posterior biceps and semitendinosus nerves (4 volleys, 320 Hz, 3 x threshold; $4PBST_{3T}$). 1, before; 2, after 2 mg/kg βCPG, intravenous. A2 and B2, 4 min after βCPG when the gastrocnemius-soleus monosynaptic reflex (GS MSR) had been reduced to 30% of its control value; C2, 2 min after βCPG when the GS MSR had been reduced to 55% of its control value. Voltage calibration: 0.1 mV.

DISCUSSION

Muscimol should be a valuable model compound for investigating the structural and conformational requirements of bicuculline-sensitive, postsynaptic GABA receptors. This isoxazole is a potent bicuculline-sensitive depressant of spinal neurones (Johnston, Curtis, de Groat and Duggan, 1968; Curtis, Duggan, Felix and Johnston, 1971), and yet it is only a weak non-competitive inhibitor of GABA uptake by rat brain slices (Johnston, 1971). Certain structural and conformational requirements are essential before a molecule will successfully interact with bicuculline-sensitive, postsynaptic GABA receptors (see Section I and Curtis and Watkins 1960, 1965). Consequently, the GABA-like postsynpatic action of muscimol is presumably a consequence of the conspicuous structural similarities between GABA and muscimol. Unlike GABA, however, the muscimol molecule is relatively rigid and the conformational restrictions of this compound and structurally related isoxazoles can be used to further analyse the bicuculline-sensitive GABA receptor.

Structural modification of muscimol produced compounds that were either weaker than or similar in potency to GABA, the potency depending upon the nature of the structural changes. Elongation of the side chain of muscimol with a further methylene group, or substitution of an aliphatic hydrogen atom with a methyl group gives compounds X and IV, respectively. The depressant actions of these two compounds were weaker than that of muscimol,

but comparable in potency with those of their open chain analogues, δ- and γ-aminovaleric acids (Curtis and Watkins, 1960 and see also Krogsgaard-Larsen, Johnston, Curtis, Game and McCulloch, 1975). Further increasing the size of the alkyl group in compound IV produced compounds V and VI which were weaker agonists than compound IV. On the other hand, substitution of the aromatic protons of muscimol and compound X with alkyl groups greatly reduced the effectiveness of these compounds as neuronal depressants, the derivatives VII, VIII and XI being very weak or inactive. These derivatives have substituents in the 4-position, and the 4-positions of muscimol and compound X are isosteric with the α-methylene group of GABA and δ-aminovaleric acid, respectively. Smythies (1974) has recently postulated that the α-methylene group of GABA is involved in lipophilic binding in the GABA-receptor complex. Thus the complete rigidity of the isoxazole ring may mean that substituents in the 4-position prevent interaction with the GABA receptor.

The interaction of trans-4-aminocrotonic acid with bicuculline-sensitive postsynaptic receptors further supports previous suggestions regarding the structural similarities of bicuculline, GABA, muscimol and 4-aminotetrolic acid (Curtis, Duggan, Felix and Johnston, 1971; Beart, Curtis and Johnston, 1971). Trans-4-aminocrotonic acid and muscimol represent relatively rigid and extended conformations of GABA and the potent GABA-like effects of these compounds on spinal neurones may indicate that the low energy conformations of the compounds reflects the conformations of GABA required

for interaction with the GABA receptor. Molecular orbital calculations predict an extended conformation of muscimol in the conservative state (Kier and Truit, 1970), whereas muscimol adopts a twisted conformation in the solid state as shown by X-ray crystallography. The intramolecular $O(2)^-$ and $N(1)^+$ distances from X-ray analysis and molecular orbital calculations, however, are in approximate agreement; 5-6 Å from calculations and 5.772 Å as analysed (Brehm, Hjeds and Krogsgaard-Larsen, 1972). These and other X-ray crystallographic studies have indicated structural similarities between muscimol (Brehm, Hjeds and Krogsgaard-Larsen, 1972) and GABA (Tomita, Higshi and Fujiwara, 1973) and between the hydrochlorides of trans-4-aminocrotonic acid and GABA (Tomita, 1971). In addition to the structural similarities, however, the relative rotational freedom of these agonists may be an important factor, since it is likely that formation of the agonist-receptor complex involves a process of conformational selection which will influence the kinetics and thermodynamics of the interaction.

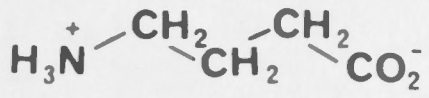
X-Ray analysis of compound X and 3-hydroxy-5 (3-aminopropyl)isoxazole (compound XVI) are also available (Brehm, Krogsgaard-Larsen and Hjeds, 1974), and despite the different aliphatic side chains the distances between the charged centres in muscimol and compound X in the solid state conformation are very similar, but distinctly different from the corresponding distance in compound XVI which was a very weak depressant. These structural findings, plus the GABA-like depressant effects of muscimol and H2AI, indicate that the intramolecular distance between

the zwitterionic centres of GABA agonists may be of importance for their postsynaptic activity. The appropriate distances for muscimol (5.772 Å, X-ray analysis; Brehm, Hjeds and Krogsgaard-Larsen, 1972) and H2AI (5.979 Å, X-ray analysis; Brehm, Krogsgaard-Larsen and Hjeds, 1974) are within the ranges of those accessible to the conformationally restricted GABA-like agonists, trans-4-aminocrotonic (4.9-6.1 Å, Dreiding stereomodels) and 4-aminotetrolic acids (5.2-5.8 Å, Dreiding stereomodels Beart, Johnston and Uhr, 1972).

Due to the difficulty experienced in obtaining precise relative potencies of the aminocyclohexane derivatives, a consequence of their weak activities as depressants, it is only possible to obtain limited information in terms of their structure-activity relationships. A structure-activity analysis is further limited due to the number of conformational modes that are available to the aminocyclohexane derivatives. It is probably significant, however, that the two analogues in which the distance between the zwitterionic centres is restricted to less than 5 Å (Dreiding stereomodels; Beart, Johnston and Uhr, 1972), cis- and trans-2-amino-cyclohexane-1-carboxylic acid were the weakest members of the series. Furthermore, the cis-2- derivative, which probably has the closest zwitterionic centres, was found to be very weak or inactive in these tests.

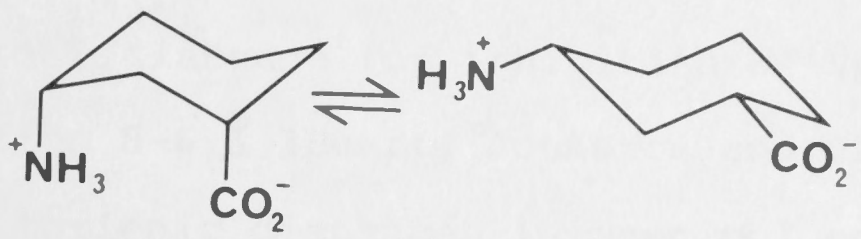
Cis- and trans-3-aminocyclohexane-1-carboxylic acids were the two most potent depressants in the aminocyclohexane series, and although Table 3 suggests that they were of similar potency, the cis-3- derivative

was the more potent compound in a direct comparison on the two neurones that were depressed by the cis-3- derivative. There appears to be no ready explanation for the excitation of 6 of the remaining 10 neurones on which cis-3-aminocyclohexane-1-carboxylic acid was tested. The chair conformation of cis-3- and trans-3-aminocyclohexane-1-carboxylic acids are probably the preferred conformations of these compounds and they are illustrated in Fig. 11. On the basis of pK measurements, Hewgill and Jefferies (1955) have concluded that DL-cis-3-aminocyclohexane-1-carboxylic acid existed in aqueous solution predominantly in the diequatorial conformation (Fig. 11), in this conformation the distances between the zwitterionic centres are from approximately 4.8 Å to 6.2 Å (Dreiding steremodels; Beart, Johnston and Uhr, 1972). The energy barrier between the two conformers, however, might be quite small and either conformer may be the active one. The interzwitterionic distance in the diaxial conformer of cis-3-aminocyclohexane-1-carboxylic acid is from 3.9 to less than 1.5 Å (Dreiding stereomodels, Beart, Johnston and Uhr, 1972), a distance which is less than the maximum distance found in the very weak or inactive cis-2- derivatives. Cis-3- and trans-3-aminocyclohexane-1-carboxylic acids are inhibitors of GABA uptake by rat brain slices (Beart, Johnston and Uhr, 1972) and may thus be substrates for that transport system, a factor which could complicate the estimation of their relative potencies, but these two compounds were more potent depressants than the trans-2- derivatives which did not inhibit uptake (Beart, Johnston and Uhr, 1972). It is likely that the cis-2- derivatives are not substrates for the GABA uptake system and thus



GABA

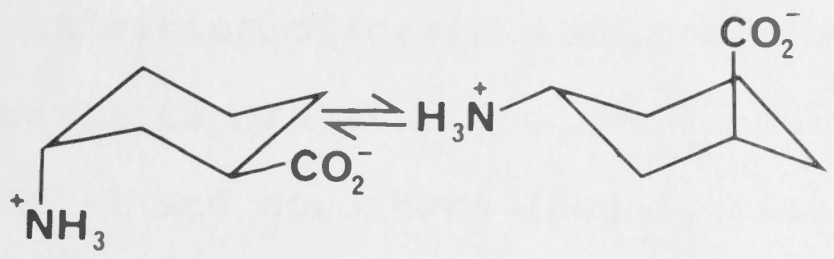
(fully extended)



DL-cis-3-aminocyclohexane-

1-carboxylic acid

(diaxial) (diequatorial)



DL-trans-3-aminocyclohexane-

1-carboxylic acid

Fig. 11:

The structures of GABA, (±)-cis- and (±)-trans-3-aminocyclohexane-1-carboxylic acids. Only the chair forms of the two cyclohexane derivatives are shown.

they may attain higher concentrations in the extracellular space than the cis-3- derivatives, after electrophoretic ejection.

The results with the aminocyclohexane-1-carboxylic acids as neuronal depressants suggest that the optimal charge separation for depressant activity is from 5-6 Å. A similar conclusion was reached when these compounds were studied as inhibitors of GABA uptake and the optimum interzwitterionic distance for inhibition of uptake was found to be from 5-6 Å (Beart, Johnston and Uhr, 1972). The interzwitterionic distances in muscimol and H2AI fall within the 5-6 Å range (see above), but of these two compounds only muscimol is a weak inhibitor of GABA uptake (Johnston, 1971).

The activities of certain aminocyclohexane-carboxylic acids as depressants of rat hippocampal neurones have been reported by Segal and coworkers (Segal, Sims, Maggiora and Smissman, 1973). Similar difficulties in estimating relative potencies were apparently encountered in this work in which the trans-2- and cis-3- conformers were reported to be the most potent GABA analogues. A relative assessment using submaximal responses was apparently not made on each neurone, and the significance of the quoted potencies may thus be questioned. The depressant action of cis-3-aminocyclohexane-1-carboxylic acid on rat hippocampal neurones was reported to be blocked by bicuculline, but no details of the testing conditions were given (Segal, Sims, Maggiora and Smissman, 1973).

The selective enhancement of the depressant action of electrophoretically administered GABA on spinal neurones by nipecotic acid and compound XIII is suggestive of the importance of the uptake process in terminating the depressant action of this amino acid. Of the compounds listed in Table 2 only XIII and the structurally related cyclic amino acids perhydro-1,2-oxazine-6-carboxylic acid and nipecotic acid are significant inhibitors of high affinity GABA uptake (Krogsgaard-Larsen and Johnston, 1975), a system which has been shown to have considerable structural and conformational specificity (Iversen and Neal, 1968; Iversen and Johnston, 1971; Beart and Johnston, 1973; Beart, Johnston and Uhr, 1972; Johnston, 1971). The intramolecular distance between the charged centres in nipecotic acid and compound XIII is 4.236-4.758 Å (X-ray analysis, see Krogsgaard-Larsen, Johnston, Curtis, Game and McCulloch, 1975) and 4.4 Å (Dreiding stereomodels, Krogsgaard-Larsen and Hjeds, 1974), respectively. Muscimol (Johnston, 1971) and compound IV (Krogsgaard-Larsen and Johnston, 1975) are potent neuronal depressants, but very weak inhibitors of GABA uptake, and hence it is evident from Table 2 that the structural and conformational requirements for interaction of GABA analogues with the uptake system and postsynaptic receptors must be different.

Trans-4-aminocrotonic acid was demonstrated to be a bicuculline-sensitive depressant of spinal neurones with a potency similar to that of GABA. Hence this compound substitutes for GABA at the postsynaptic receptor, in the GABA uptake system (Johnston, Curtis, Beart, Game,

McCulloch and Twitchin, 1974) and it is also a substrate for the GABA catabolising enzyme GABA:2-oxoglutarate aminotransferase (Beart and Johnston, 1973). The folded GABA analogue, cis-4-aminocrotonic acid was not a substrate for GABA:2-oxoglutarate aminotransferase, does not inhibit GABA uptake by rat brain slices (Johnston, Curtis, Beart, Game, McCulloch and Twitchin, 1974) and is a weak bicuculline-insensitive depressant of spinal neurones. Similarly, neither cis-2- nor trans-2-aminocyclohexane-1-carboxylic acid inhibited the uptake of GABA by rat brain slices (Beart, Johnston and Uhr, 1972) and both these compounds were weak or inactive as depressants of spinal neurones. It would thus appear likely that extended conformations of GABA are important with respect to the interaction of GABA and the specific macromolecules which mediate GABA uptake and neuronal depression, however, the biological activities of the compounds in Table 2 have indicated that the two processes have slightly different structural and conformational requirements.

It would seem reasonable to assume that after electrophoretic ejection from solutions at pHs from 2-3.5 the GABA analogues that have been investigated in these experiments take up ionic forms that depend only upon the local pH. Electrophoretically or pressure-ejected GABA from a neutral solution (Krnjević and Phillis, 1963), or electrophoretically ejected GABA from solutions at either pH 2.5-3 or pH 11-12 (Curtis, Phillis and Watkins, 1959) depresses central neurones. The isoelectric point for GABA at 35^{o}C is pH 7.5 (see King, 1954), and consequently

the predominant form in feline extraneuronal fluid would be the zwitterion. The dissociation constants of the ionizable groups in the GABA analogues of Table 4, appear to be distributed over an appreciable range, and are thus possibly somewhat less important than structural and conformational factors.

TABLE 4 Ionisation constants for GABA and some conformationally restricted analogues of GABA

Compound	pK values (20°C)		Reference
GABA	4.04	10.71	King, 1954
cis-4-Aminocrotonic acid	3.53	9.84	1
trans-4-Aminocrotonic acid	3.55	9.46	2
cis-3-Aminocyclohexane-1-carboxylic acid	3.70	-	3
trans-3-Aminocyclohexane-1-carboxylic acid	3.85	-	3
4-Aminotetrolic acid	1.80	8.34	4
3-Hydroxy-5-(2-aminoethyl)-isoxazole (X)	5.12	9.46	5
3-Hydroxy-5-(3-aminopropyl)-isoxazole (XVI)	5.37	10.36	5
3-Hydroxy-5-aminomethyl-isoxazole (muscimol)	4.78	8.43	Eugster, 1969
Piperidine-3-carboxylic acid (nipecotic acid)	3.86	10.28	6
5,6,7,8-Tetrahydro-4H-isoxazolo[4,5-d]azepin-3-ol (XII)	4.84	9.20	5
4,5,6,7-Tetrahydroisoxazolo-[4,5-c]pyridin-3-ol (XIII)	4.33	9.06	5

References: 1, Johnston, Curtis, Beart, Game, McCulloch and Twitchin, 1974; 2, Beart, Johnston and Uhr, 1972; 3, Hewgill and Jefferies, 1955; 4, Beart and Johnston, 1972; 5, Brehm, Krogsgaard-Larsen and Hjeds, 1974; 6, Krogsgaard-Larsen and Johnston, 1975.

The relatively weak and slow depressant action of cis-4-aminocrotonic acid, which could not be blocked by either bicuculline or strychnine, is of interest in view of the similar weak and apparently BMC-insensitive depressant effects of compounds V and VI which are slow in onset. In addition, no evidence was found for the interaction of βCPG with the bicuculline-sensitive receptors associated with GABA-mediated synaptic inhibition. Davies and Watkins (1974) have also reported a bicuculline-insensitive depression of cerebral cortical neurones by βCPG, and of the various GABA 'receptors' present in mammalian central nervous tissue, βCPG possibly only interacts with those of GABA:2-oxoglutarate aminotransferase (Curtis, Game, Johnston and McCulloch, 1974). Interaction of βCPG with glycine receptors seems unlikely in view of the minimal effect of this compound on Renshaw cells and the strychnine-insensitive depression of spinal interneurones. Unlike βCPG, cis-4-aminocrotonic acid appeared to be equally potent as a depressant of the firing of Renshaw cells and spinal interneurones. Isoxazoles V and VI were not tested on Renshaw cells.

The reduction <u>in vitro</u> by βCPG of high affinity L-glutamate uptake by rat brain slices (Curtis, Game, Johnston and McCulloch, 1974) does not account for the depression of neuronal firing by βCPG. Furthermore, antagonism at excitant amino acid receptors or a reduced release of excitatory transmitters does not readily account for the depressant effects of βCPG. If glycine and GABA receptors are not involved in this depressant action, then it would seem possible that the excitability

change results from interaction with another type of neuronal receptor, which may be associated with the operation of an inhibitory transmitter other than glycine or GABA. This transmitter may be structurally related to the phenylethylamine moiety of βCPG rather than to the GABA portion of the molecule, but the lack of correlation between the depressant effects of βCPG and noradrenaline, a phenylethylamine related compound (Fig. 9), suggests that the hypothesised transmitter is unlikely to be noradrenaline.

Although not tested directly in the present experiments, βCPG probably has an action on spinal motoneurones similar to that observed with interneurones, pyramidal tract neurones and Purkinje cells, since relatively low doses administered systemically markedly reduced spinal monosynaptic reflexes (see also Bein, 1972). The relative ineffectiveness of electrophoretically administered βCPG as a depressant of Renshaw cell firing was unexpected, although an earlier report indicated that βCPG usually had no action on the firing of bulbar reticular neurones which were sensitive to glycine and GABA (Hösli, Tebēcis and Haas, 1971). When this compound was systemically administered in doses sufficient to reduce spinal monosynaptic reflexes there was no apparent change in the rhythmic wave of inverted phase ('Renshaw ripple') recorded from the dorso-lateral surface of the spinal cord in response to antidromic stimulation of the ventral roots and believed to be due to Renshaw cell discharges (Eccles, Fatt and Koketsu, 1954). Hence, neither electrophoretically nor systemically administered

βCPG was shown to significantly affect the excitability of Renshaw cells.

Preliminary experiments have indicated an increase in membrane conductance and decreased excitability when βCPG was administered electrophoretically near spinal motoneurones (Curtis, Game, Johnston and McCulloch, 1974). Such a post-synaptic effect, however, does not explain the reduction of excitatory postsynaptic potentials of these cells in the absence of alterations in either inhibitory postsynaptic potentials or membrane excitability reported to follow systemic administration of βCPG (Pierau and Zimmermann, 1973), observations which suggest a presynaptic effect of this agent at excitatory synapses on motoneurones, but not at those on spinal inhibitory neurones. The absence of a significant effect of βCPG on the 'quadriceps notch' which was recorded from the dorso-lateral surface of the spinal cord in response to stimulation of the quadriceps nerve, and is possibly generated by excitation of quadriceps Ia inhibitory interneurones, may indicate that inhibitory interneurones, like Renshaw cells, are relatively insensitive to this compound, but this would need to be tested at the cellular level.

When administered systemically βCPG simultaneously reduced monosynaptic reflexes and DRPs, generally accepted as a measure of primary afferent depolarisation (PAD) (Schmidt, 1971), a process which is associated with presynaptic inhibition of spinal reflexes. GABA appears to participate in the development of PAD; evidence for this participation includes reduction of PAD and the

inhibitory effect on reflexes of incoming volleys by picrotoxinin and bicuculline (Schmidt, 1971; Curtis, Duggan, Felix and Johnston, 1971; Levy and Anderson, 1972), but direct evidence for terminal depolarisation has not been obtained in the cat. When applied topically to the spinal cord, GABA and 3-amino-1-propanesulphonic acid depressed DRPs and dorsal root reflexes and thus may have depolarised primary afferent fibres (Eccles, Schmidt and Willis, 1963). Electrophoretically administered GABA, however, depressed terminal excitability (Curtis and Ryall, 1966a). These topically administered amino acids, like systemically administered βCPG, may reduce DRPs not only by depolarising primary afferent terminals, but also by depressing the interneurones on the pathway that is responsible for generating PAD.

The structural basis of the antagonism of the inhibitory effect of glycine by the isoxazoles XII and XIII and by nipecotic acid is not clear. Interestingly, however, this activity, which is relatively weak compared with that reported for the specific glycine antagonist strychnine (Curtis, Duggan and Johnston, 1971) seems to increase with increasing substitution of the amino group of the model compounds.

In summary, it should be emphasised that conclusive evidence concerning the 'active conformations' of GABA cannot be derived from this analysis. Only a limited number of model compounds has been investigated, and it is conceivable that these compounds interact with a limited population of synapses operated by GABA. Some of the compounds that were investigated were only weak depressants

of spinal neurones, and consequently the sensitivities of these effects to bicuculline have not been clearly established, and the conclusions drawn from these particular compounds must be treated with caution. The present evidence favours extended conformers of GABA as the active ones at bicuculline-sensitive postsynaptic synapses, and there are indications that the macromolecules that mediate the postsynaptic GABA-like depressant action and the high affinity uptake of GABA have different structural and conformational requirements. The distances between the charged centres of GABA agonists and inhibitors of GABA uptake required for biological activity generally appear to be within different but apparently rather narrow ranges.

IV. BICUCULLINE ANALOGUES AS GABA ANTAGONISTS AND ANTICHOLINESTERASES

The convulsant pthalide isoquinoline alkaloid bicuculline (Manske, 1933) has proved of considerable value in studying inhibitory processes in the mammalian central nervous system mediated by GABA. When administered electrophoretically near single neurones, (+)-bicuculline selectively reduces the inhibitory effects of GABA (or GABA-like amino acids - see Curtis, Hösli and Johnston, 1968; Curtis, Duggan, Felix and Johnston, 1971; Curtis, Duggan, Felix, Johnston and McLennan, 1971) with little or no reduction of the effects of glycine (and glycine-like amino acids) or noradrenaline. Selective antagonism of this type has been demonstrated in the spinal cord (Curtis, Duggan, Felix and Johnston, 1971) and the following supraspinal regions: cuneate nucleus (Kelly and Renaud, 1973), medullary reticular formation (Tebēcis, Hösli and Haas, 1971; Tebēcis, 1973), hypoglossal nucleus (Duggan, Lodge and Biscoe, 1973), lateral vestibular nucleus (Curtis, Duggan, Felix, Johnston and McLennan, 1971), hypothalamus (Nicoll and Barker, 1971; Dreifuss and Mathews, 1972), thalamic ventrobasal complex (Curtis, Duggan, Felix, Johnston and McLennan, 1971), lateral geniculate nucleus (Curtis and Tebēcis, 1972), cerebellar interpositus nucleus (Kawaguchi and Ono, 1973), septal nuclei (McLennan and Miller, 1974), cerebellar, cerebral and hippocampal cortices (Curtis, Duggan, Felix, Johnston and McLennan, 1971), and the olfactory bulb (Felix and McLennan, 1971; Nicoll, 1971). Furthermore, bicuculline (administered intravenously or electrophoretically) has

been shown to suppress various synaptic inhibitions that are suspected of being mediated by GABA on the basis of neurochemical and neuropharmacological evidence, and many of which are found in those previously quoted regions of the central nervous system in which bicuculline has been demonstrated to be a specific GABA antagonist (see Curtis and Johnston, 1974a, 1974b).

Certain difficulties have been experienced in the experimental use of bicuculline, largely because a saturated solution at pH 3, as used in microelectrophoretic experiments, contains approximately 5 mM bicuculline hydrochloride which is even less soluble at a more physiological pH (Curtis, Duggan, Felix and Johnston, 1971; Pong and Graham, 1972). Some controversy has thus arisen regarding bicuculline-GABA antagonism in the mammalian central nervous system, and much of this controversy is undoubtedly due to improper preparation of bicuculline solutions (see Curtis and Johnston, 1974b). Some controversy has also arisen as a result of the difficulties associated with the interpreation of the non-equilibrium 'dose-response' measurements employed by Hill and Simmonds (1973) (see also Straughan, Neal, Simmonds, Collins and Hill, 1971). This type of evaluation of microelectrophoretic antagonism was discussed in the introduction to this thesis and the matter is further considered in the next section (V).

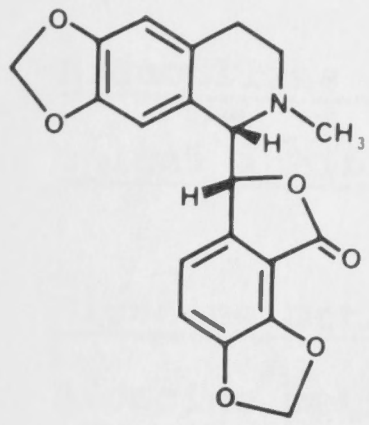
Despite the published evidence indicating that the convulsant action of bicuculline and a number of structurally related alkaloids is related to selective antagonism of the inhibitory action of GABA-like amino

acids on central neurones, many of which are insensitive to acetylcholine (see Curtis and Johnston, 1974b), the suggestion has been made that the "physiological" effects of bicuculline may be more closely related to "some aspect of the function of the cholinergic system than to effects on the GABA system" (Svenneby and Roberts, 1973). This suggestion was based on the observation that bicuculline hydrochloride and bicuculline methochloride (N-methyl-bicuculline) competitively inhibit brain acetylcholine-sterase activity in vitro, but have no effect on the enzyme associated with the synthesis (L-glutamate-1-carboxylase) and degradation (GABA-2-oxoglutarate aminotransferase) of GABA by a crude mitochondrial fraction of mouse brain. It is, however, very likely that the receptors of GABA-metabolising enzymes and of GABA transport sites differ in their susceptibility to antagonists from that of receptors associated with the postsynaptic action of GABA: inhibitors of GABA enzymes and transport (Curtis, Duggan and Johnston, 1970) appear not to block the inhibitory action of this amino acid (see also Section III).

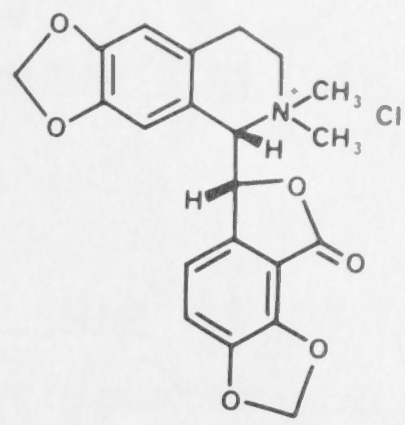
Bicuculline excites central neurones, especially those which can also be excited by acetylcholine (Curtis, Duggan, Felix and Johnston, 1971; Curtis, Duggan, Felix, Johnston and McLennan, 1971). Such excitation could be associated with the inhibition of cholinesterases and the enhancement of synaptically released acetylcholine. Concentrations of these alkaloids adequate to suppress the inhibitory action of GABA, however, do not invariably excite neurones and bicuculline can excite non-

cholinoceptive spinal interneurones. Thus it is likely that other factors are involved in the excitation, including reduction of 'background' GABA-mediated inhibition and direct excitation.

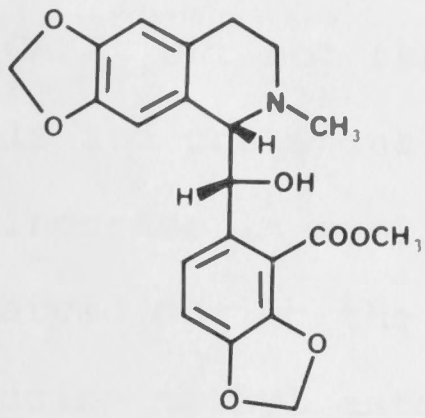
A series of bicuculline analogues has thus been investigated for a specific GABA antagonist with physiochemical properties that might be more suitable than those of bicuculline for microelectrophoresis. In addition this investigation sought to obtain structure-activity relationships regarding the convulsants that were investigated (Fig. 12). A study was made of the effects of these compounds, which are all structurally related to bicuculline, upon the sensitivity of neurones to the inhibitory amino acid GABA. In view of the excitant action of bicuculline a study was also made on spinal Renshaw cells to determine the relative importance of any anti-cholinesterase activity of bicuculline hydrochloride and bicuculline methochloride.



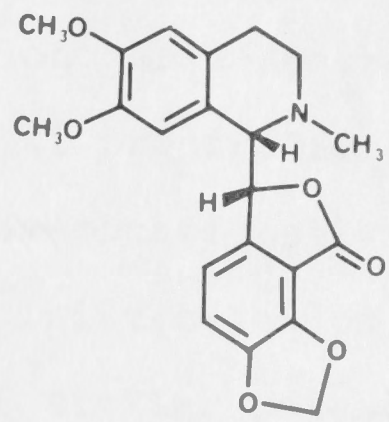
BICUCULLINE



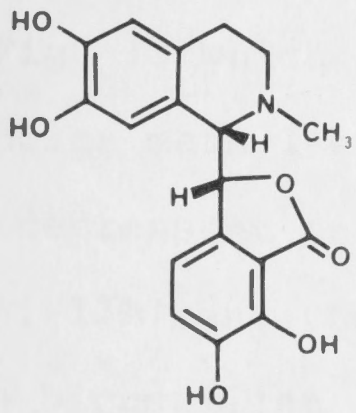
BICUCULLINE
METHOCHLORIDE



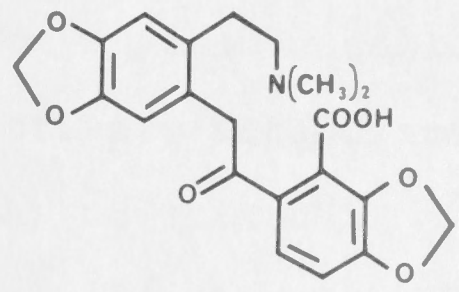
BICUCINE
METHYL ESTER



CORLUMINE



BICUCULLINE
TETRAPHENOL



N-METHYL-BICUCULLEINE

Fig. 12:

Structures of some bicuculline analogues.

RESULTS

(1) Bicuculline analogues as antagonists of GABA and spinal inhibitions

(a) Bicucine methyl ester and corlumine

Bicucine methyl ester when administered with cationic currents (30-60 nA) from solutions of less than 5 mM in 165 mM NaCl at pH 3 consistently and selectively blocked or reduced the inhibitory action of GABA, but not that of glycine, on three Renshaw cells and one spinal interneurone (1 preparation). An increase in firing frequency was occasionally observed during the continuous administration of bicucine methyl ester which was of similar potency to bicuculline (5 mM in 165 mM NaCl, pH 3) when the two compounds were compared as GABA antagonists on the same neurones. One of these comparisons is illustrated in Fig. 13 which is a recording from a Renshaw cell. Bicucine methyl ester (30 nA) selectively antagonised the depressant action of GABA (5 nA) on this cell (Fig. 13A) and the antagonism was similar to that seen with bicuculline (30 nA) (Fig. 13B). Corlumine (15 mM in 165 mM NaCl, pH 3) was also similar in action and potency to bicuculline when compared on three Renshaw cells (1 preparation) with similar currents (30-100 nA). The selective antagonism of GABA by corlumine (60 nA) is compared with that due to bicuculline (60 nA) on the Renshaw cell depicted in Fig. 14. In addition, when administered intravenously in an other spinal cord preparation, corlumine (1-2.5 mg/kg)

RENSHAW CELL

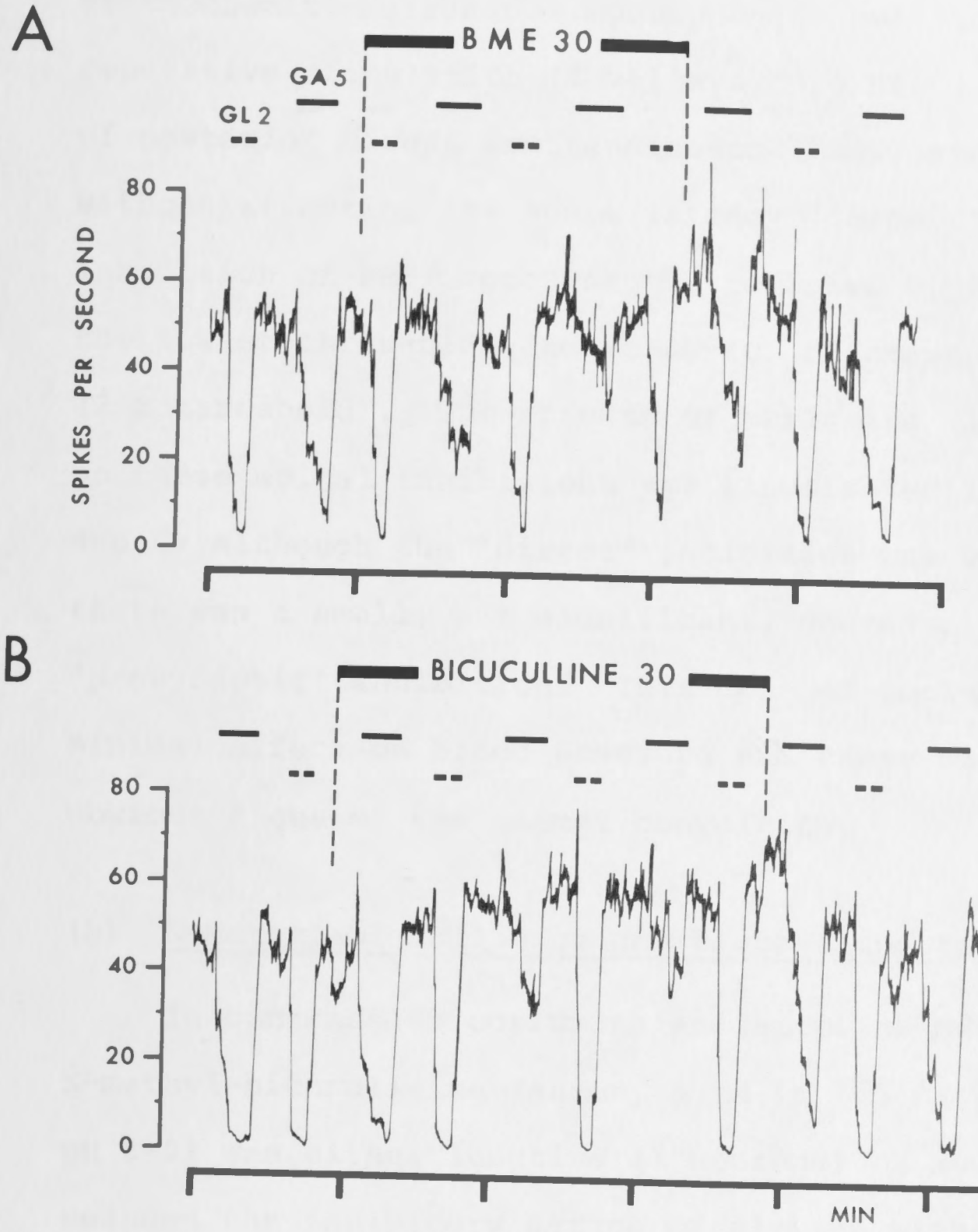


Fig. 13:

Effects of bicucine methyl ester (BME) and bicuculline on the depressant actions of glycine and GABA. The firing rate of a Renshaw cell excited by electrophoretically ejected DL-homocysteate (DLH, 7 nA) was depressed by glycine (GL, 2 nA) and GABA (5 nA), indicated by the broken and solid horizontal lines, respectively. A, responses before, during and after the continuous administration of BME (approximately 5 mM in 165 mM NaCl) by a current of 30 nA; B, responses before, during and after the continuous administration of bicuculline (5 mM in 165 mM NaCl) by a current of 30 nA. The solid horizontal bars and vertical broken lines indicate the periods of continuous administration. Ordinates: firing frequency in spikes per second. Abscissae: time in minutes.

reduced the prolonged (presynaptic) inhibition of the gastrocnemius-soleus (GS) monosynaptic reflexes by repetitive stimulation (4 volleys, 320 Hz, 3 x threshold) of posterior biceps semitendinosus (PBST) afferents, without affecting the short latency ('direct') inhibition of PBST monosynaptic reflexes by impulses in the lowest threshold quadriceps (Q) afferent fibres (2 x threshold). The effects of corlumine (2 mg/kg) on these spinal inhibitions are illustrated in Fig. 14C and D: although the 'direct' inhibition was unaffected, there was a small, but significant, decrease in 'presynaptic' inhibition. This dose of corlumine had minimal effect on blood pressure and there were no obvious signs of the animal convulsing.

(b) <u>N-Methyl-bicuculleine and bicuculline tetraphenol</u>

In contrast to corlumine and bicucine methyl ester, N-methyl-bicuculleine (anion, 5 mM in 165 mM NaCl, pH 8-9) was either inactive (I neurone) or selectively reduced the inhibitory action of glycine without affecting the action of GABA (2 Renshaw cells, 1 interneurone) when administered with anionic currents of 60-200 nA (1 preparation). Bicuculline tetraphenol (7.5 mM in 165 mM NaCl, pH 3) was a non-selective antagonist of GABA and glycine. Although bicuculline and strychnine were selective antagonists of GABA and glycine respectively, on the 5 neurones tested (3 interneurones, 2 Renshaw cells; 2 preparations), bicuculline tetraphenol at all 'dose' levels (5-60 nA) was non-selective and appeared to be more potent as a glycine antagonist than a GABA antagonist. Fig. 15A

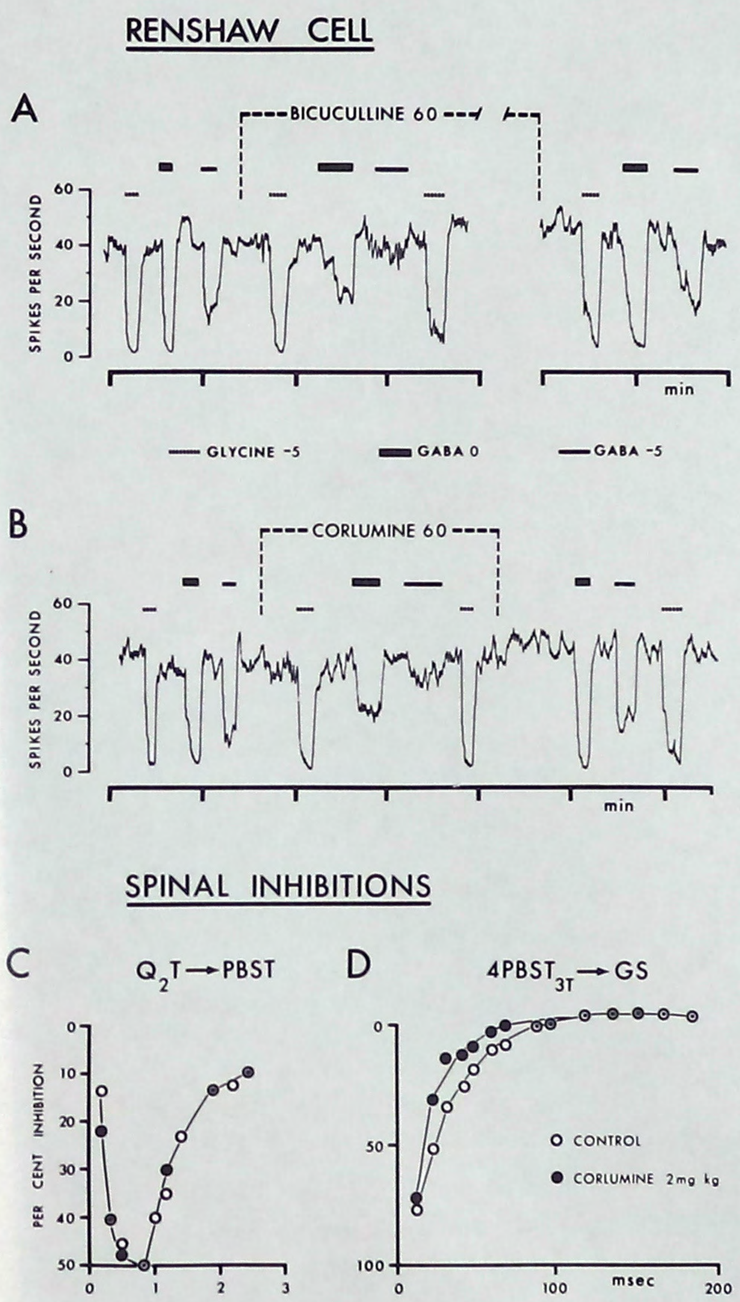


Fig. 14:

Effects of corlumine and bicuculline on the depressant actions of glycine and GABA, and the effect of corlumine on the inhibition of spinal monosynaptic reflexes recorded from the first sacral ventral roots. The Renshaw cell (A and B) was excited by the continuous electrophoretic administration of DL-homocysteate (DLH, 5 nA) and depressed by glycine (-5 nA) and GABA (0 nA = removal of retaining current, and -5 nA), indicated by the broken and solid horizontal lines respectively. A, responses before, during and after the continuous administration of bicuculline (5 mM in 165 mM NaCl) for 4.5 min; B, responses before, during and after the continuous administration of corlumine (15 mM in 165 mM NaCl), 60 nA. The horizontal broken bars and vertical broken lines indicate periods of continuous electrophoretic administration. C, PBST (posterior biceps semitendinosus) monosynaptic reflex inhibited by one Q (quadriceps) volley (2 x threshold); D, GS (gastrocnemius soleus) reflex inhibited by 4PBST volleys (3 x threshold, 320 Hz). The curves in C and D relate percent inhibition (ordinates) to the interval (abscissae) between the inhibitory and testing volleys, each symbol indicating the mean value of 4 observations of the reflex (area). Open circles: control observations. Filled circles: C, 20 min after a total dose of corlumine of 2 mg/kg intravenous, 1 mg/kg of which had been administered 8 min earlier. D, 12 min after the same doses of corlumine as in C.

illustrates the effect of two 'dose levels' of bicuculline tetraphenol on a spinal interneurone that was responding to the depressant action of glycine and GABA. Bicuculline tetraphenol (20 nA) reduced the depressant action of glycine (12 nA) to less than half the control response whilst the action of GABA (18 nA) was only slightly reduced. Bicuculline tetraphenol (35 nA) almost abolished the glycine and GABA responses, but there was rapid and complete recovery when administration of bicuculline tetraphenol ceased. Bicuculline methochloride (see later) (BMC: 5 nA) had minimal effect on the glycine response and reduced the GABA response to about 30% of the control (Fig. 15B). Strychnine (12 nA) was similarly a selective antagonist on this interneurone, almost abolishing the depressant action of glycine (12 nA) and having no effect on the response to GABA (18 nA) (Fig. 15C). Unlike bicuculline methochloride, corlumine, bicucine methylester and N-methyl bicuculleine, bicuculline tetraphenol had no significant effect upon the firing frequency of the cells on which it was tested.

(c) Bicuculline methochloride

Bicuculline methochloride, ejected with currents of 10-80 nA from either a 10 mM solution in 165 mM NaCl (pH 3.5) or from a 100 mM aqueous solution, reduced the sensitivity of spinal neurones to GABA; the influence on the action of glycine was minimal (4 Renshaw cells, 6 interneurones; 4 preparations). The effects were readily reversible and the substance appeared to be more potent than bicuculline on the

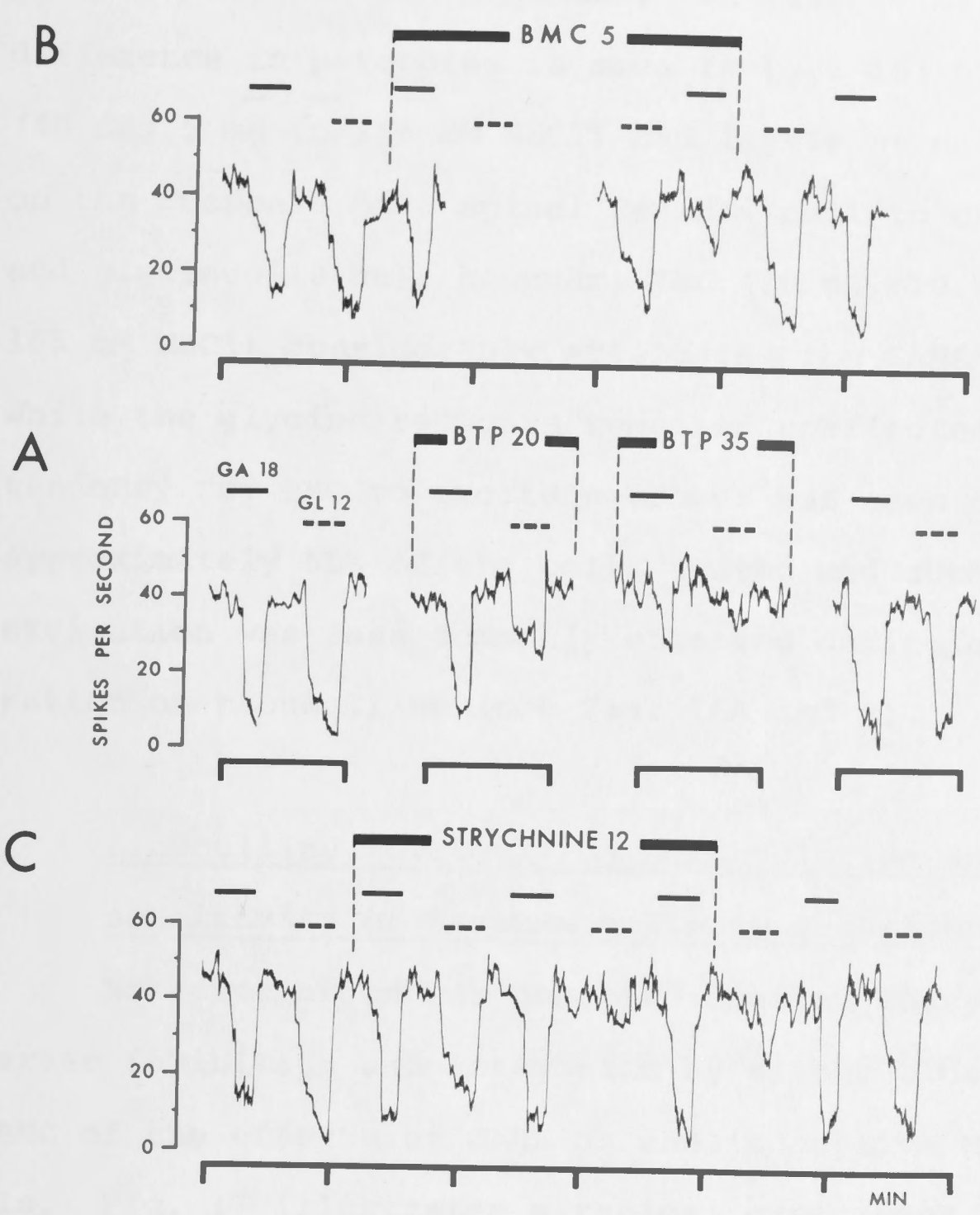


Fig. 15:

Effects of bicuculline tetraphenol (BTP), bicuculline methochloride (BMC) and strychnine on the depressant actions of GABA and glycine. The rate of firing of a dorsal horn interneurone excited by DLH (0 nA) was depressed by electrophoretically administered GABA (GA, 18 nA) and glycine (GL, 12 nA) indicated by solid and broken horizontal lines, respectively. A, responses before, during and after the ejection of BTP (7.5 mM in 165 mM NaCl) by successive currents of 20 and 35 nA; B, before, during and after the ejection of BMC (10 mM in 165 mM NaCl) with a current of 5 nA; C, before, during and after the ejection of strychnine (2 mM in 165 mM NaCl) with a current of 12 nA. The solid horizontal bars and broken vertical lines indicate periods of continuous electrophoretic ejection.

basis of comparison of equally effective electrophoretic currents on six neurones. An example of the difference in potencies is seen in Fig. 16: bicuculline (40 nA, 5 mM in 165 mM NaCl) had little or no effect on the response of a spinal Renshaw cell to GABA (5 nA) and glycine (10 nA), however, BMC (10 nA, 10 mM in 165 mM NaCl) considerably attenuated the GABA response while the glycine response remained unaffected. A tendency for BMC to excite neurones was seen in approximately 50% of the cells tested and such an excitation was less commonly observed during administration of bicuculline (see Fig. 16A and B).

(2) Bicuculline, bicuculline methochloride and the sensitivity of Renshaw cells to acetylcholine

No association was observed between acetylcholinesterase inhibition and antagonism by either bicuculline or BMC of the effects of GABA on cholinoceptive Renshaw cells. Fig. 17 illustrates a typical experiment in which BMC in a concentration (35 nA) sufficient to block electrophoretically administered GABA (5 nA) enhanced the background firing rate of the cell, but did not accentuate the effectiveness of acetylcholine with respect to that of DLH. Similar observations were made upon this cell using bicuculline (150 nA). The type of experiment illustrated in Fig. 17A and B was performed on a total of eight Renshaw cells (three preparations) with identical results. The effects of intravenous bicuculline, and physostigmine salicylate, on Renshaw cell responses to DL-homocysteate and acetylcholine were compared on three

RENSHAW CELL

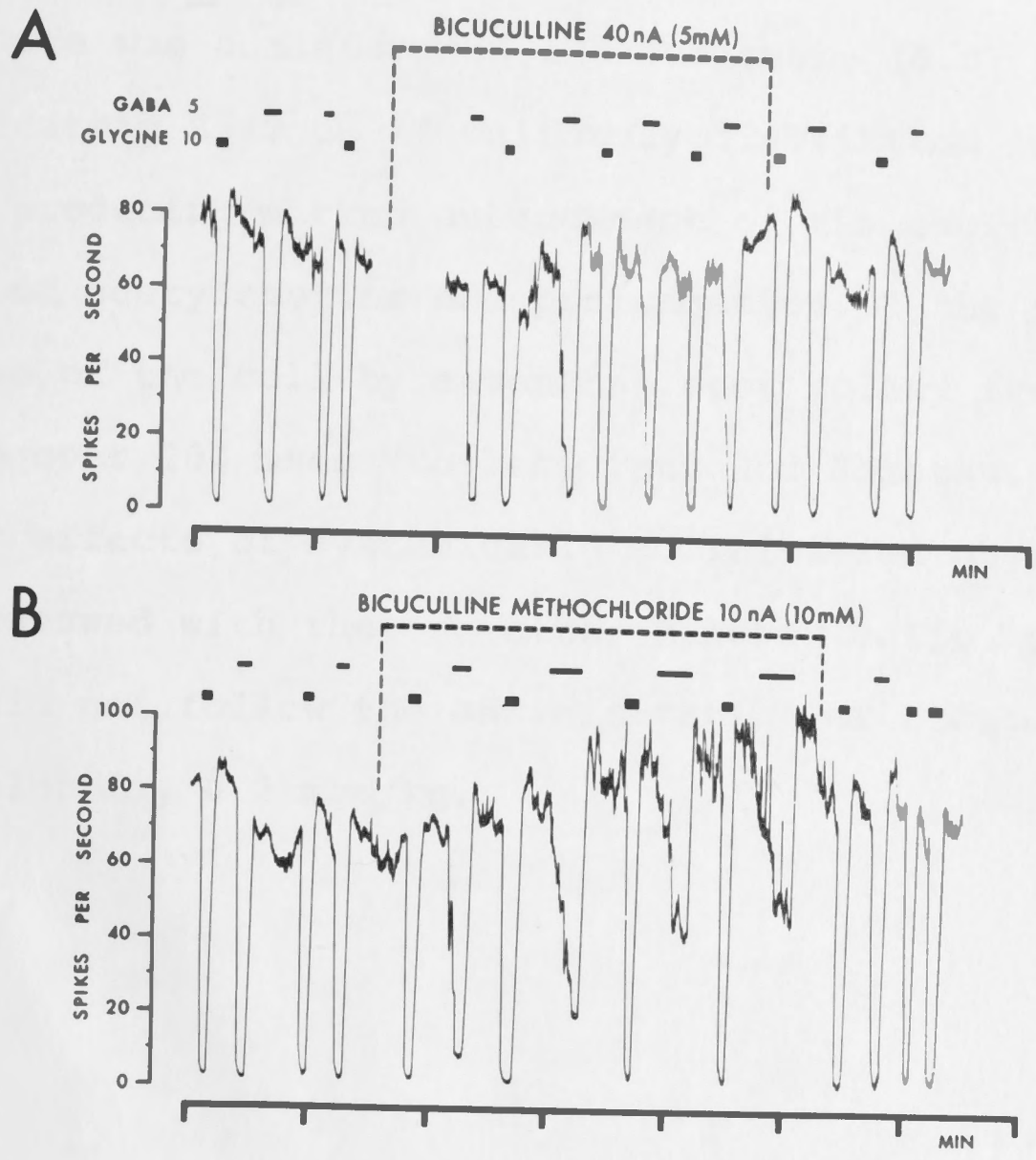


Fig. 16:

Effects of bicuculline and bicuculline methochloride (BMC) on the depressant actions of glycine and GABA. The rate of firing of a Renshaw cell, excited by the continuous electrophoretic administration of DLH (4 nA) was depressed by GABA (5 nA) and glycine (10 nA), indicated by the respective horizontal lines. A, before, during and after the continuous administration of bicuculline (5 mM in 165 mM NaCl) with a current of 40 nA; B, before, during and after the administration of BMC (10 mM in 165 mM NaCl) with a current of 10 nA. The broken horizontal bars and broken vertical lines indicate periods of continuous electrophoretic administration.

Renshaw cells (one cell per preparation). Fig. 17C is a continuation of the experiment with the same Renshaw cell that is illustrated in Fig. 17A and B. Physostigmine salicylate was administered intravenously (0.05 µgm/kg, approximately 0.12 µM if uniformly distributed in body water) producing marked enhancement of the excitatory action of acetylcholine and prolongation of the synaptic response of the cell by a ventral root volley from 40-50 msec to over 200 msec (Eccles, Fatt and Koketsu, 1954). Similar effects of systemically administered physostigmine were observed with the two other Renshaw cells, effects which did not follow the administration of bicuculline hydrochloride, 0.2 mgm/kg.

RENSHAW CELL

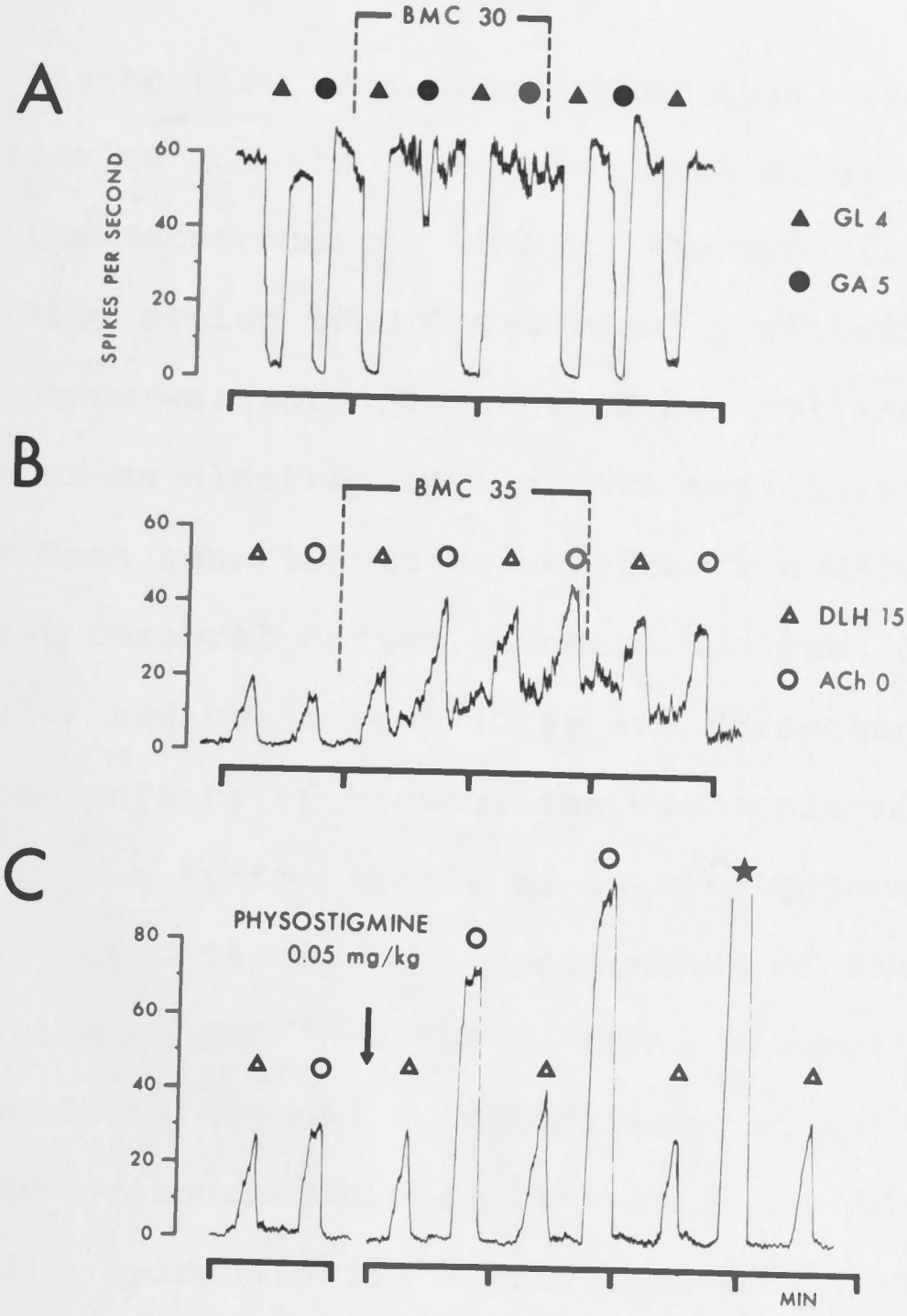


Fig. 17:

Effects of bicuculline methochloride (BMC, A and B) and physostigmine (C) on the action of amino acids and acetylcholine (ACh) on a Renshaw cell. A, effect of electrophoretically administered BMC (5 mM in 165 mM NaCl) with a current of 30 nA during the time of the solid horizontal bar, on the inhibition of DLH (15 nA) maintained firing by glycine (GL, 4 nA for 11 sec) and GABA (GA, 5 nA for 11 sec); B, effect of electrophoretic BMC (35 nA) on firing by DLH (15 nA for 11 sec) and ACh (0 nA = removal of retaining current for 11 sec); C, effect of intravenous physostigmine salicylate (0.5 mg/kg) on the firing in response to DLH (15 nA) and ACh (0 nA). The star indicates firing in excess of 120 spikes per sec.

DISCUSSION

Of the bicuculline analogues that were tested the most effective and also the most water soluble was bicuculline methochloride (BMC). The specific GABA antagonistic action of BMC was readily reversible, and the substance was more potent than bicuculline hydrochloride as an electrophoretic GABA antagonist. BMC has recently been reported to be an effective GABA antagonist in the rat cerebral cortex, cuneate nucleus (Collins and Hill, 1974) and brain stem (Dray and Straughan, 1974). The higher potency of bicuculline methochloride with respect to the hydrochloride as an electrophoretic antagonist of GABA may be a consequence of the complete dissociation of BMC into the N-methyl bicuculline cation and chloride in aqueous solution, whereas only approximately 10% of the protonated bicuculline would be present in a bicuculline hydrochloride solution at 25°C (Andrews and Johnston, 1973). In addition, data from molecular orbital calculations and proton magnetic resonance spectra suggests a different range of preferred rotational conformers for the N-methyl bicuculline cation and the protonated bicuculline cation, for rotation around the C_1-C_9 bond in bicuculline and its salts is not free (Andrews and Johnston, 1973). The convulsant dose of BMC (intravenous) for mature mice was approximately 12 mg/kg (bicuculline, 0.2 mg/kg), while the dose (intraperitoneal) for 10 day old rats was 10 mg/kg (bicuculline, 1 mg/kg) (Johnston, Beart, Curtis, Game, McCulloch and MacLachlan, 1972). The relative ineffectiveness of BMC as a systemically

administered convulsant is possibly a consequence of a blood-brain barrier and/or metabolic degradation. BIcuculline methiodide ('N-methyl bicuculline'), which antagonises GABA in the frog spinal cord (Davidoff, Silvey, Kobetz and Spira, 1973) is a more potent convulsant than bicuculline when administered intracisternally to rats (Pong and Graham, 1972). At the crayfish (Pacifastacus leniusculus) neuromuscular junction and stretch receptor bicuculline and BMC were approximately equipotent (McLennan, 1973).

Bicucine methyl ester and corlumine were similar in action and potency to bicuculline when compared electrophoretically. The effect of corlumine on the inhibition of spinal reflexes was also investigated. Short latency spinal inhibitions are sensitive to the glycine antagonist strychnine, and strychnine-like compounds. Thus 'direct' inhibition is reversibly reduced by relatively low concentration of intravenously administered strychnine (Bradley, Easton and Eccles, 1953), and there is a large body of evidence which indicates that short latency spinal inhibitions are mediated by glycine (see Curtis and Johnston, 1974a). In contrast, spinal 'presynaptic' or prolonged inhibition is insensitive to strychnine, but reduced by picrotoxinin (Eccles, Schmidt and Willis, 1963), which has no effect on spinal 'direct' inhibition, and by bicuculline (Curtis, Duggan, Felix and Johnston, 1971). It is thus likely that this type of prolonged inhibition involves the release of GABA from the terminals of a particular type of inhibitory interneurone (Schmidt, 1971; Curtis and Johnston, 1974a).

By investigating the effects of convulsants on spinal inhibitions it is possible to obtain information regarding the possible GABA or glycine antagonistic properties of different compounds. The results with electrophoretically administered corlumine were thus confirmed by an investigation of the effects of this convulsant on spinal inhibitions.

The effect of systemically administered corlumine in slightly reducing the prolonged inhibition of gastrocnemius reflexes by repetitive stimulation of the posterior biceps semitendinosus nerves was weaker than that reported for bicuculline (Curtis, Duggan, Felix, and Johnston, 1971) and systemically administered corlumine is a less potent convulsant than bicuculline, the convulsant dose for corlumine in rabbits being 3 mg/kg (intravenous: Rice, 1938) whilst that for bicuculline is 0.2 mg/kg (Welch and Henderson, 1934). Additional tests would be required to obtain an accurate comparison of the effects of bicuculline and corlumine on spinal inhibitions.

Electrophoretically administered corlumine did not significantly antagonise the inhibitory action of glycine, and systemically administered corlumine had no effect on the direct inhibition of posterior biceps semitendinosus reflexes by volleys in the lowest threshold afferent fibres of the ipsilateral quadriceps nerve, an inhibition that is reduced by strychnine (Bradley, Easton and Eccles, 1953). In contrast, N-methyl-bicuculleine was a glycine antagonist and is thus similar to the reported actions of strychnine (Curtis, Duggan and

Johnston, 1971) and laudanosine (Curtis, Duggan, Felix and Johnston, 1971).

The structures of corlumine and bicucine methyl ester and their abilities to antagonise GABA are consistent with the models of GABA-bicuculline antagonism suggested by Curtis, Duggan, Felix and Johnston (1970) or Steward, Player, Quilliam, Brown and Pringle (1971) (see Section I). The property of bicuculline tetraphenol to antagonise GABA is also consistent with these models, however, those features of the bicuculline molecule that are described by the bicuculline-GABA antagonism model are obviously not the only features of importance. Opening the two dimethoxy rings in the bicuculline molecule to form bicuculline tetraphenol produces a less selective GABA antagonist, and hence these two rings appear to be important structure-activity features, provided the non-specific antagonism demonstrated for bicuculline tetraphenol is not due to a non-receptor action of this compound. Bicuculline tetraphenol is structurally related to the glycine antagonist, laudanosine, but many apparently structurally unrelated compounds are glycine antagonists.

The excitation of central neurones by (+)-bicuculline and some of its analogues, in particular (+)-bicuculline methochloride appears not to be related to GABA antagonism for similar excitation occurs with (-)-bicuculline methochloride which like (-)-bicuculline is not a GABA antagonist ((-)-BMC: Collins and Hill, 1974; Hill, Simmonds and Straughan, 1974; (-)-bicuculline: Johnston and Curtis, 1973). Picrotoxin, bicuculline and

strychnine directly excite isolated lobster axons by causing a depolarisation which is associated with a rise in membrane resistance (Freeman, 1973), the minimal effective concentration of picrotoxin to produce this excitation was 5×10^{-5} - 1×10^{-4} M and the minimal effective concentration of bicuculline was similar. This concentration range is similar to that used in various crustacean preparations to antagonise the effect of GABA or to reduce synaptic inhibition (see McLennan, 1970 - crayfish, Eustacus armatus; Shank, Pong, Freeman and Graham, 1974 - lobster, Homarus americanus), but the concentrations produced after systemic administration of convulsant doses of bicuculline to mammals are probably well below this range. (The convulsant dose of bicuculline for mammals is approximately 0.2 mg of the hydrochloride per kg body weight (Wellch and Henderson, 1934), equivalent to approximately 10^{-6} M if uniformly distributed in body fluids.) Similarly, doses of bicuculline that are sufficient to reduce bicuculline-sensitive spinal, cerebellar and cerebral inhibitions (0.5-0.6 mg/kg: Curtis, Duggan, Felix and Johnston, 1971; Curtis, Duggan, Felix, Johnston and McLennan, 1971) are unlikely to produce the direct excitant effect that was observed in vitro by Freeman (1973). The possibility still exists, however, that the excitation of central neurones by bicuculline and some of its analogues is due to a non-synaptic action of these compounds. Such a non-synaptic action would not necessarily detract from the value of bicuculline as a GABA antagonist, for in properly designed electrophoretic experiments, non-bicuculline-sensitive depressants are

used as controls and the firing frequency of neurones can usually be maintained constant, so that control responses to depressants are obtained with the neurones firing at the same frequency as that during the administration of bicuculline. The demonstration that there are bicuculline-insensitive inhibitions (Curtis, Duggan, Felix and Johnston, 1971) is evidence that the reduction of inhibitions by bicuculline is not due to neuronal excitation.

The experiments on Renshaw cells excited with alternate pulses of acetylcholine and DL-homocysteate provided no support for the proposal by Svenneby and Roberts regarding the participation of central cholinergic systems in the convulsant action of bicuculline and related alkaloids (and it seems more reasonable to consider that this action primarily involves antagonism of GABA-like amino acids). Physostigmine, a competitive antagonist of acetylcholinesterase with an apparent K_i of 0.06 μM (Augustinsson and Nachmansohn, 1949) and thus some 100 times more potent <u>in vitro</u> than bicuculline hydrochloride (K_i 65 μM; Svenneby and Roberts, 1973), clearly diminished the activity of this enzyme <u>in vivo</u> and greatly enhanced the sensitivity of Renshaw cells to acetylcholine. Such an effect was not apparent with systemic doses of bicuculline hydrochloride adequate to influence GABA-mediated central inhibitions (Curtis and Johnston, 1974b). Miller and McLennan (1974) have recently reported that electrophoretically administered BMC enhanced the excitant effect of electrophoretic pulses of acetylcholine on acetylcholine sensitive

neurones of the rat septal nuclei, but a control agonist was not used to determine the specificity of the effect.

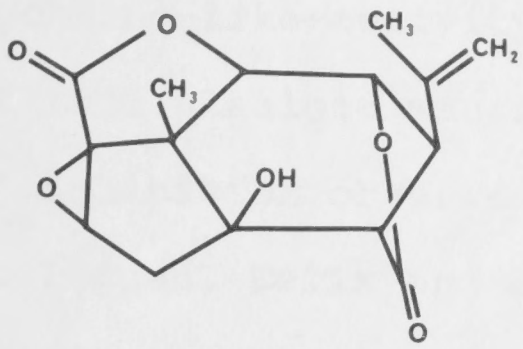
The above experiments have shown that the central effects of bicuculline and BMC cannot be adequately explained on the basis of a weak anticholinesterase effect, measured in vitro. The K_i values for BMC (25 μM) and bicuculline hydrochloride (65 μM) as competitive inhibitors of mouse brain acetylcholinesterase (Svenneby and Roberts, 1973) would appear to be of the wrong order of magnitude of that expected by a potent centrally acting drug when its mechanism of action is tested in vitro. For comparison, the apparent binding constants of strychnine (0.3 μM) to rat spinal synaptic membrane fractions (Young and Snyder, 1973) is several orders of magnitude less than the K_i values measured by Svenneby and Roberts. Similar systemic concentrations of strychnine hydrochloride and bicuculline hydrochloride are effective on glycine and GABA mediated inhibitions, respectively. Thus doses of strychnine of 0.01-0.2 mg/kg (0.025-0.5 μM) antagonise glycine mediated central inhibitions (Curtis and Johnston, 1974b) and 0.05-0.1 mg/kg (0.15-0.3 μM) of bicuculline hydrochloride suppresses the Purkinje cell inhibition of Deiters' neurones in the cat (Curtis, Duggan and Felix, 1970). In view of the in vivo doses of strychnine, bicuculline (and physostigmine) required for central effects, a K_i of the order of 0.01-0.1 μM would be expected for a binding of bicuculline measured in vitro, if it reflects the central mechanism of this convulsant. Evidence has been provided for a sodium dependent binding of GABA by synaptosomal fractions of rat cerebellar cortex

which can be competitively inhibited by bicuculline, and may represent binding to the postsynaptic GABA receptor (Peck, Schaeffer and Clark, 1973). These particular experiments, however, indicated an apparent K_i of 80 μM for bicuculline hydrochloride, of the same order of magnitude as the K_i values for BMC and bicuculline hydrochloride as competitive inhibitors of mouse brain acetylcholinesterase, a binding which has been shown to be unrelated to the main central action of bicuculline. Accordingly the binding measured by Peck and co-workers seems unlikely to represent post-synaptic GABA receptors.

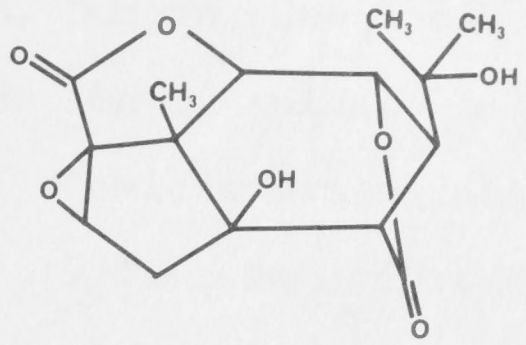
V. SOME CONVULSANTS AS ANTAGONISTS OF INHIBITORY AMINO ACIDS

There are many different mechanisms of action that could explain the convulsions that are produced by centrally acting convulsant substances (see Watkins, 1968), and the mechanisms of action of relatively few of the known convulsant poisons have been elucidated. It is of fundamental pharmacological interest to determine the mechanism of action of a convulsant, and if the mechanism of action is found to be selective blockade of the action of an inhibitory transmitter substance on the postsynaptic membrane, then the convulsant could be a useful pharmacological antagonist - useful for determining the role of inhibitory substances and in structure-activity relationship studies. For these reasons the central actions of some potential antagonists of the inhibitory amino acids, glycine and GABA, have been investigated. The compounds investigated included three convulsants that are structurally related to picrotoxin, together with penicillin and d-tubocurarine.

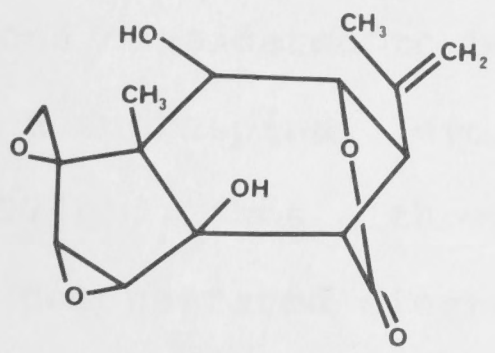
Although the chemically related compounds dendrobine and picrotoxinin (Fig. 18) are both convulsants when administered to mammals, there is evidence to indicate that these substances differ in their central modes of action. Dendrobine, which occurs in the ornamental orchid Dendrobium nobile and is an active constituent of the Chinese herbal preparation Chin-shih-hu (Chen, K.K. and Chen, A.L., 1935; Inubushi, Sasaki, Tsuda, Yasui, Konita, Matsumoto, Katarao and Nakano, 1964;



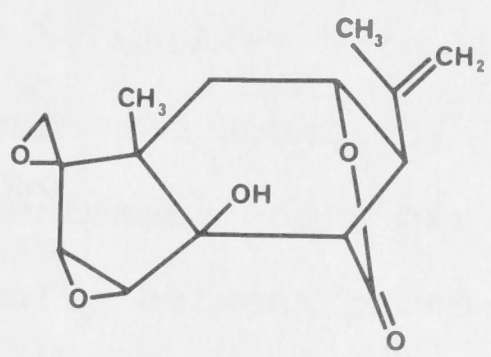
PICROTOXININ



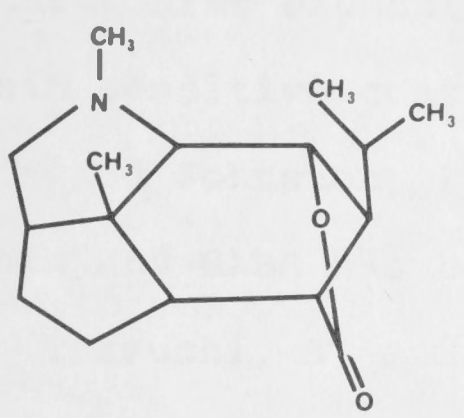
PICROTIN



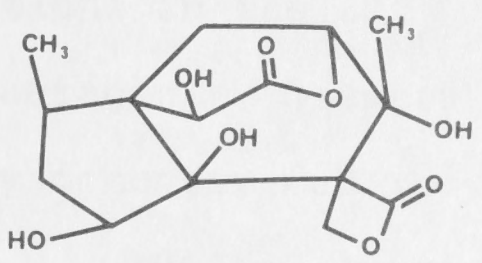
TUTIN



CORIAMYRTIN



DENDROBINE



SHIKIMIN

Fig. 18:

Structures of some convulsants chemically related to picrotoxinin.

Porter, 1967), is a glycine antagonist when administered electrophoretically near feline spinal neurones (Curtis, Duggan, Felix and Johnston, 1971). Further evidence of the strychnine-like activity (Curtis, Duggan, Johnston, 1971) of this alkaloid was the diminution of spinal 'direct' inhibition observed after systemic administration (Curtis, Duggan, Felix and Johnston, 1971). In contrast, picrotoxinin, the active principle of picrotoxin (see Porter, 1967) which is isolated from plants of the moon-seed family, Menispermaceae, reduces a number of central inhibitions considered to be mediated by GABA at both the spinal and supraspinal level (see Curtis and Johnston, 1974a, 1974b). Thus although consistent antagonism has not been demonstrated electrophoretically between picro-toxinin and GABA, picrotoxin is generally considered to be a selective GABA antagonist in mammals, particularly as the GABA antagonist bicuculline reduces many of these picrotoxinin sensitive central inhibitions in the cat (see Curtis and Johnston, 1974b) and antagonism between picrotoxinin and GABA has been clearly demonstrated in crustacea (Takeuchi, A. and Takeuchi, N., 1969). In view of the differences in the central actions of dendrobine and picrotoxinin, a study was made of three other structurally related convulsants, coriamyrtin, shikimin and tutin (Fig. 18). Shikimin has been isolated from the ripe fruit of the evergreen tree Shikimin (Kajimoto, Fujimori and Hirota, 1955; Kajimoto, Ota, Fujimori, Harada and Hirota, 1955), and tutin occurs in the berries of the indigenous New Zealand tutu plant, Coriaria arborea (Fastier, 1971; Fitchett and Malcolm, 1909; Marshall,

1906). Coriamyrtin was first isolated from the European Coriaria myrtifolia (see Porter, 1967) and was later found to be a close structural analogue of tutin, differing only in the absence of the secondary hydroxyl group of tutin. Tutin and coriamyrtin are reported to have very similar central effects (Marshall, 1912), there appears, however, to be no published reports concerning its action on central inhibitory processes. Picrotoxinin, tutin and coriamyrtin have similar convulsant doses in mice (Jarboe, Porter and Buckler, 1968), but the convulsive dose of shikimin in mice is about 100 times that of picrotoxinin, the convulsions being preceeded by general paralysis (Kajimoto, Ota, Fujimori, Harada and Hirota, 1955). Picrotin, not investigated in these experiments, is 50-times less potent than picrotoxinin as a convulsant (Jarboe, Porter and Buckler, 1968).

The epileptogenic action of penicillin on the mammalian cerebral cortex is well documented: after topical, intracerebral or systemic administration there is abnormal and paroxysmal activity which may lead to major seizures (Ajmone-Marsan, 1969; Gloor, 1969; Prince, 1969). When administered electrophoretically (40 nA) near single neurones of the cerebral cortex, penicillin reputedly enhanced the amplitude of certain excitatory synaptic potentials (Walsh, 1971). Prolonged depolarising postsynaptic potentials have been recorded from motoneurones after penicillin was administered topically to the feline spinal cord (Kao and Crill, 1972). On the basis of antagonism by penicillin of the depolarising effect of GABA on dorsal root terminals of the amphibian

spinal cord, Davidoff (1972a) suggested that the convulsant properties of penicillin may be associated with antagonism of inhibitions mediated by GABA within the mammalian central nervous system. Hence a study was made of the action of electrophoretically administered penicillin upon the inhibition of spinal and cortical neurones by GABA and glycine, and a comparison made between penicillin and bicuculline, a known GABA antagonist in these regions of the cat central nervous system (see Curtis and Johnston 1974a, 1974b).

d-Tubocurarine is a convulsant when administered topically to the cerebral cortex (Chang, 1953; Feldberg, Malcolm and Darian-Smith, 1957), a property which is not common to neuromuscular blocking agents (Banerjee, Feldberg and Georgiev, 1970). Until recently no evidence had been published that curare suppresses inhibitory mechanisms in either the cerebral (Bhargava and Meldrum, 1969; Banerjee, Feldberg and Georgiev, 1970) or hippocampal (Feldberg and Lotti, 1970) cortices. Hill and co-workers, however, have interpreted certain experiments in the feline cerebral cortex to indicate that the convulsant action of d-tubocurarine is due, at least in part, to antagonism of GABA mediated inhibition in that cortex (Hill, Simmonds and Straughan, 1972). In these experiments the effects of glycine were apparently also antagonised in both the cortex (Hill, Simmonds and Straughan, 1972) and the cuneate nucleus (Hill, Simmonds and Straughan, 1973a), nevertheless the claim was made that d-tubocurarine is a useful GABA antagonist.

Although there is now a considerable body of evidence, largely derived from microelectrophoretic experiments, that strychnine and bicuculline are adequately selective glycine and GABA antagonists, respectively, to provide reliable information regarding the probable nature of an inhibitory transmitter, doubt has been expressed, in recent publications concerning the convulsant action of d-tubocurarine (Hill, Simmonds and Straughan, 1973b; Dray and Straughan, 1974), regarding the usefulness of bicuculline as a GABA antagonist. Furthermore, the claim was made that d-tubocurarine (dTC) is not only more potent than bicuculline (and picrotoxin) as an antagonist of the inhibitory action of GABA on the firing of feline cortical neurones, but additionally is more useful "in terms of practical microiontophoresis". The proposal of Hill and co-workers (Hill, Simmonds and Straughan, 1973b) regarding the usefulness of dTC as a GABA antagonist appears to ignore the "marked excitant effect" of dTC on cortical neurones and the little, if any, selectivity demonstrated between the inhibitory actions of glycine and GABA (see also Hill, Simmonds and Straughan, 1972, 1973a). This apparent lack of specificity contrasts with previously reported observations of the selective antagonism, by bicuculline, of the inhibitory action of GABA-like amino acids upon neurones in the feline cerebral cortex, other supraspinal regions and the spinal cord (see Section IV). Consequently dTC has been examined as an amino acid antagonist in the feline spinal cord, and in particular its activity as a GABA antagonist compared with that of a more soluble bicuculline analogue,

bicuculline methochloride (BMC: Johnston, Beart, Curtis, Game, McCulloch and MacLachlan, 1972; see also Section IV of this thesis).

RESULTS

(1) Shikimin

The low aqueous solubility of shikimin prevented electrophoretic testing, but intravenous doses of 1.0 mg/kg caused dilation of the cat's pupils, salivation and oscillations in blood pressure - typical effects of an intravenously administered convulsant of the picrotoxinin type. Doses of 1.5 mg/kg, which had little or no effect on the direct inhibition of posterior biceps semitendinosus (PBST) monosynaptic reflex by impulses in the lowest threshold quadriceps (Q) afferent fibres, considerably reduced the long latency and duration inhibition of the gastrocnemius soleus (GS) monosynaptic reflex by repetitive stimulation (4 volleys, 320 Hz, 3 x threshold) of PBST afferents (2 preparations). Fig. 19A illustrates the effect of shikimin (1.0 mg/kg, intravenous) on the long latency inhibition of 4 $PBST_{3T}$ on GS. Three hours after the last dose of shikimin was administered to this preparation, 0.1 mg/kg strychnine was administered intravenously and the early (peak) inhibition that was resistant to shikimin (1.5 mg/kg, intravenous) was reduced by 50% (at the 15 msec inhibitory-testing volley interval) without altering the late inhibition.

(2) Tutin

Tutin (1.5-3.0 mg/kg, intravenous) had a similar action to that of shikimin upon prolonged (presynaptic) spinal inhibition (Fig. 19C) and, like bicuculline, reduced the dorsal root potential generated by the

afferent inhibiting PBST volley (Curtis, Duggan, Felix and Johnston, 1971). In three experiments, doses of 1.5-3.0 mg/kg did not influence the direct inhibition of PBST reflexes, although effects that are typical of intravenously administered picrotoxinin-like convulsants were usually observed (oscillating blood pressure, salivation, dilated pupils and an increase in the number of spontaneous discharges in the dorsal and ventral roots). Intravenous doses that were greater than 3 mg/kg were required to produce any reduction in the direct inhibition of PBST reflexes and Fig. 19B illustrates that 6.4 mg/kg slightly reduced this inhibition. Doses of 0.5 mg/kg or higher reduced the 'presynaptic' inhibition of GS or flexor digitorum longus (FDL) reflexes. Fig. 19C illustrates the reduction of the prolonged inhibition of the GS reflex after 3.4 mg/kg tutin, an effect that was observed simultaneously with a reduction in the early phase of the prolonged inhibition of GS reflexes. The alteration in both the early and late phase of this inhibitory curve (Fig. 19C) suggests that as well as reducing the inhibitory effect of GABA, tutin may also influence that of glycine. These actions of tutin were readily apparent in microelectrophoretic studies carried out on 9 dorsal horn interneurones (5 preparations), particularly when comparisons were made with the action of bicuculline methochloride (5 neurones). These neurones were either firing spontaneously or were activated by the continuous electrophoretic ejection of DL-homocysteate. Electrophoretically administered tutin (50 mM in 165 mM NaCl, 5-10 nA) selectively and reversibly antagonised

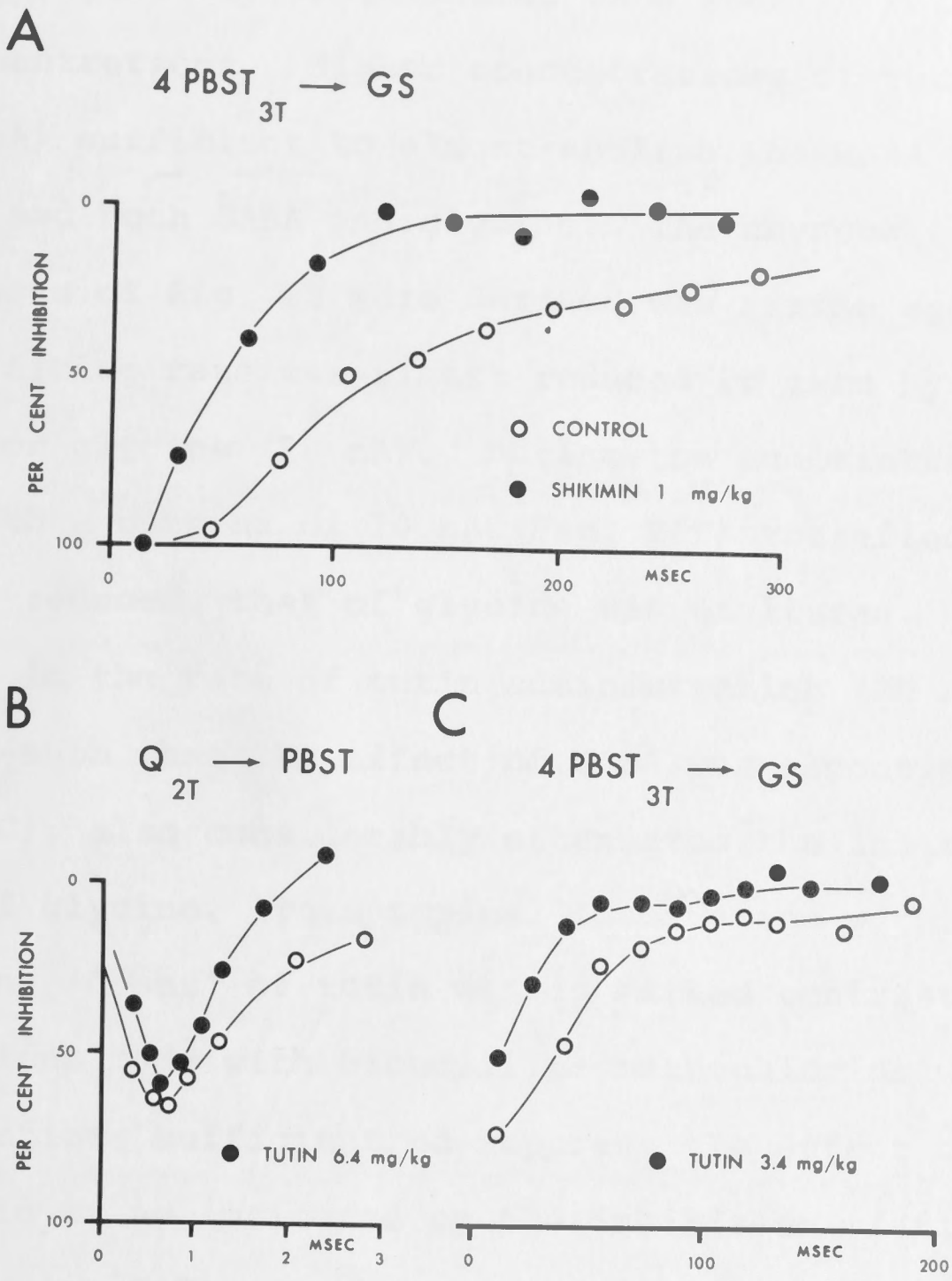


Fig. 19:

Effects of shikimin (A) and tutin (B, C) on the inhibition of spinal monosynaptic reflexes recorded from the first sacral ventral roots. A and C, gastrocnemius-soleus (GS) reflexes, inhibited by 4 PBST volleys (3 x threshold, 320 Hz), B, PBST reflexes inhibited by one quadriceps (Q) volley (2 x threshold). The curves relate per cent inhibition (ordinates) to the interval (abscissae) between the inhibitory and testing volleys, each symbol indicating the mean value of four observations of the reflex (area). Open circles: control observations. Filled circles: A, 3 min after a total dose of shikimin of 1.0 mg/kg intravenous, 0.5 mg/kg of which had been administered 22 min earlier; C, 30 min after tutin 3.4 mg/kg intravenous; B, 30 min after an additional dose of 3.0 mg/kg administered 15 min after C.

electrophoretically administered GABA when in relatively low concentrations. Higher concentrations of tutin (10-40 nA) sufficient to almost abolish the GABA response, antagonised both GABA and glycine. The neurone from which the records of Fig. 20 were derived was firing spontaneously and the firing rate was almost reduced to zero by GABA (15 nA) or glycine (20 nA). During the administration of tutin with a current of 10 nA (Fig. 20B) the effect of GABA was reduced, that of glycine was unaltered. An increase in the rate of tutin administration (20 nA), however, such that the effect of GABA was almost abolished (Fig. 20C), also considerably attenuated the inhibitory effect of glycine. This typical observation with increasing 'doses' of tutin was in marked contrast to observations made with bicuculline methochloride where concentrations sufficient to suppress the effect of GABA had little or no influence on the inhibition of firing by glycine (Fig. D-E).

(3) Coriamyrtin

Coriamyrtin was tested electrophoretically on three spinal interneurones and three Renshaw cells in two preparations. The very limited supply, and the low aqueous solubility, of this compound prevented it being tested by systemic administration and also precluded an extensive investigation of its effects at the cellular level. Electrophoretic currents of the order of 20-60 nA, from an approximately 5 mM solution (a saturated solution in 165 mM NaCl), reversibly and selectively blocked the

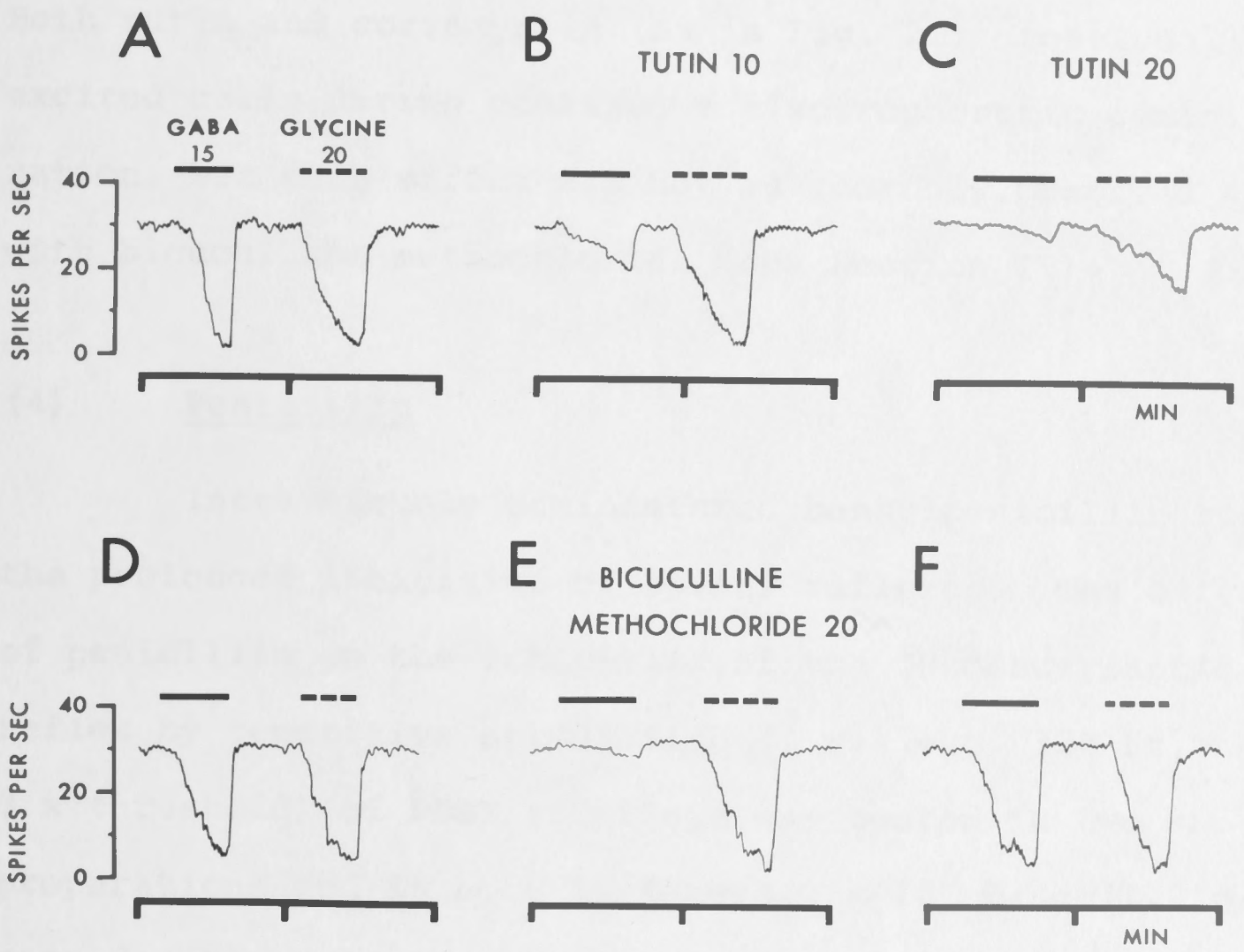


Fig. 20:

The effects of tutin (A - C) and bicuculline methochloride (D - F) on the inhibition of the spontaneous firing of a dorsal horn interneurone by GABA (15 nA) and glycine (20 nA), as indicated by the solid and broken horizontal lines respectively. A, control; B, during the electrophoretic administration of tutin (50 mM in 165 mM NaCl) with a current of 10 nA for 3.2 min; C, tutin, 20 nA for 3.8 min; D, 2 min after the termination of the tutin administration; E, during administration of bicuculline methochloride (BMC, 10 mM in 165 mM NaCl) with a current of 20 nA for 3.3 min; F, 3 min after termination of the bicuculline methochloride current.

inhibitory effect of GABA on 2 of the 3 neurones that were tested. Fig. 21 illustrates the effect of coriamyrtin (60 nA) on a Renshaw cell the DLH induced firing of which was being alternately depressed by GABA and glycine. Both tutin and coriamyrtin (as in Fig. 21) occasionally excited cells during continuous electrophoretic administration, but this effect was not as commonly observed as with bicuculline methochloride (see Section IV).

(4) Penicillin

Intravenously administered benzylpenicillin reduced the prolonged inhibition of spinal reflexes. The effect of penicillin on the inhibition of the GS monosynaptic reflex by repetitive stimulation (4 volleys, 320 Hz, 3 x threshold) of PBST afferents was tested in two preparations and in both instances 2×10^6 U/kg (1.2 g/kg) considerably reduced the prolonged (presynaptic) inhibition, but there was little or no change in the short latency inhibition of this reflex (Fig. 22A). The same dose of penicillin reduced the amplitude of dorsal root potentials generated by the afferent inhibiting PBST volley (Fig. 22B). The usual signs associated with the intravenous administration of a picrotoxinin-like convulsant were not apparent in these two unparalysed preparations - the cats' pupils remained moderately dilated and responsive to light, there was no salivation and only a slight increase in pulse pressure.

When electrophoretically administered with anionic currents of 40-250 nA, from a 0.5 M solution within four hours of preparation, penicillin reversibly reduced the

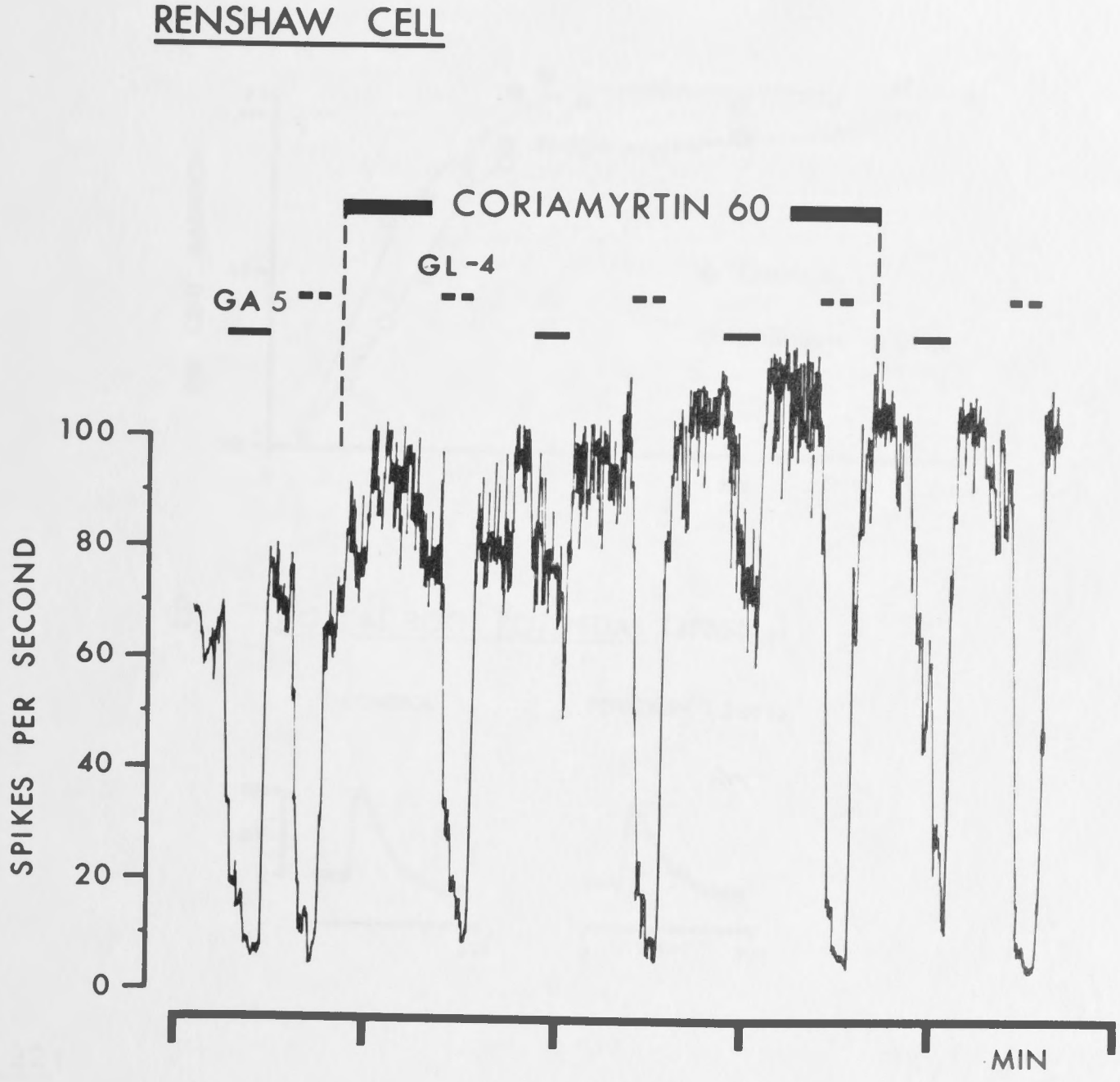


Fig. 21:

Effect of coriamyrtin on the depressant actions of GABA and glycine. The rate of DL-homocysteate (DLH, 8 nA) induced firing of a Renshaw cell was depressed by electrophoretically administered GABA (GA, 5 nA) and glycine (GL, -4 nA), indicated by the solid and broken horizontal bars, respectively: responses to GA and GL before, during and after coriamyrtin (approximately 5 mM in 165 mM NaCl), administered with a current of 60 nA. The duration of the coriamyrtin ejection is indicated by the solid horizontal bar and broken vertical lines. Because of the excitant effect of coriamyrtin the DLH current was reduced from 8 to 6 nA at the commencement of the coriamyrtin ejection period.

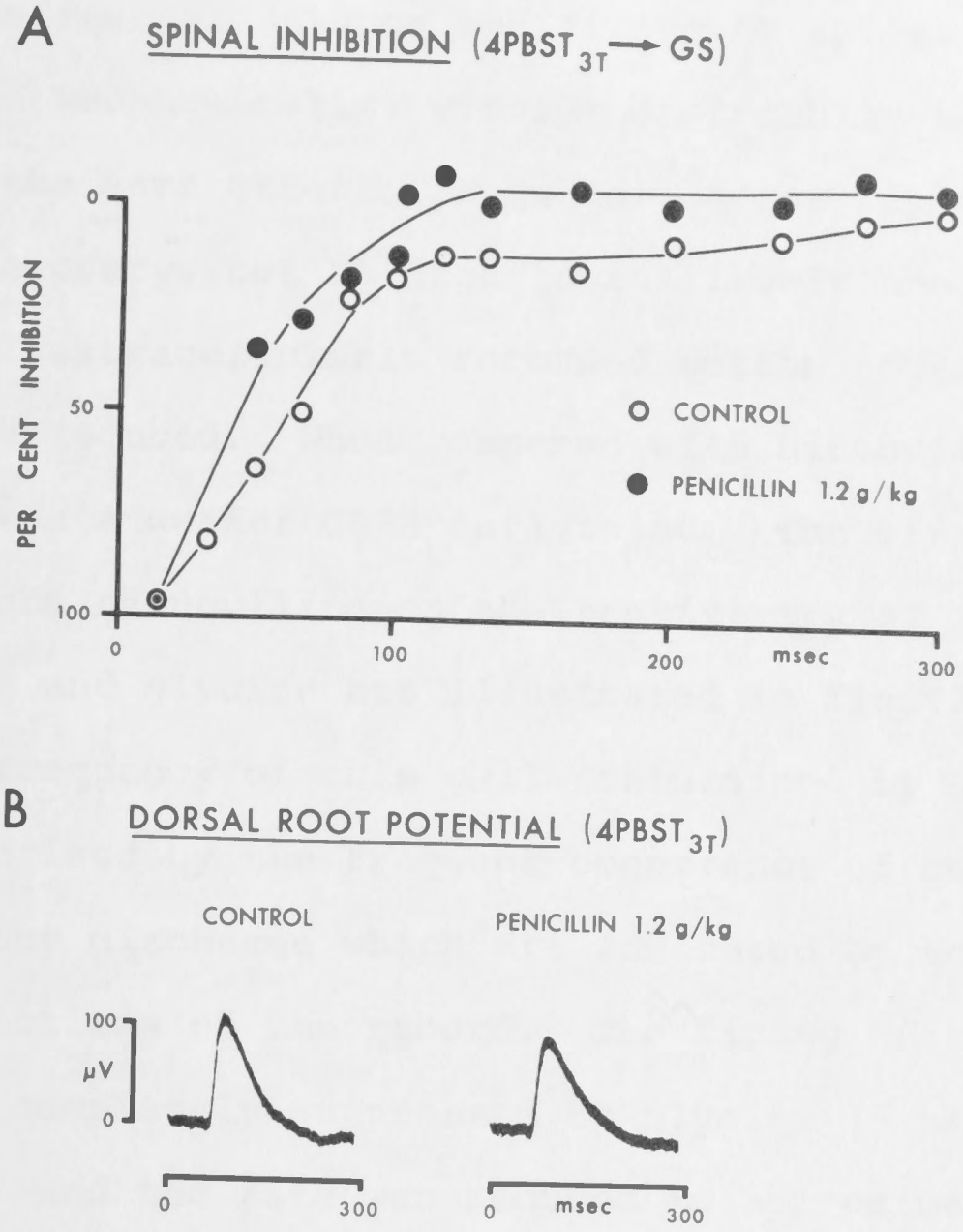


Fig. 22:

Effects of penicillin on prolonged spinal inhibition and dorsal root potentials. A, gastrocnemius soleus (GS) monosynaptic reflex recorded from the first sacral ventral roots and inhibited by repetitive stimulation (4 volleys, 320 Hz, 3 x threshold) of posterior biceps semitendinosus (PBST) afferents. The curves relate percent inhibition (ordinates) to the interval between the inhibitory and testing volleys (abscissae), each symbol indicating the mean value of 4 observations of the reflex (area). Open circles: control observations. Filled circles: 5 min after penicillin 1.2 g/kg intravenous. B, dorsal root potentials recorded with an amplifier time constant of 1 sec from the most caudal rootlet of the sixth lumbar dorsal root in response to repetitive stimulation of the PBST afferents as in A ($4PBST_{3T}$). DRP illustrated before and 12 min after penicillin 1.2 g/kg. Voltage calibration: 100 μV. Time calibration: 300 msec.

inhibitory effect of GABA on the firing of spinal interneurones and Renshaw cells, without diminishing that of glycine to the same extent. This action was rapid in both onset and recovery, but at high 'dose' levels the amplitude of extracellularly recorded action potentials tended to be reduced. When compared with bicuculline, penicillin was a weaker GABA antagonist. The effects of penicillin and bicuculline on the sensitivity of a Renshaw cell to GABA and glycine are illustrated in Fig. 23. The firing frequency of this cell, maintained by DLH (10 nA) was characterised by the frequent occurrence of bursts of high frequency discharge which are indicated by the sharp upward deflections of the record. The firing of this neurone was completely suppressed by glycine (5 nA) and GABA (10 nA) and the rate was reduced to approximately 25% by GABA (5 nA) (Fig. 23A). During the continuous administration of penicillin (120 nA) the effects of GABA were considerably attenuated (Fig. 23B), whereas those of glycine were unaltered, as is clear from a comparison with the actions of glycine (5 nA and 10 nA) immediately after termination of the penicillin ejection (Fig. 23C). This latter tracing also shows recovery of the sensitivity of the neurone to GABA and the restoration of the bursts of high frequency discharge which were suppressed during the administration of penicillin (Fig 23B). The records of Fig. 23D-F show a similar series of responses before, during and immediately after the electrophoretic administration of bicuculline (5 mM in 165 mM NaCl, 60 nA). Again, there was little or no reduction of the inhibitory effect of glycine, but considerable reduction of that of GABA. In contrast to penicillin, however, there was

accentuation rather than depression of the bursts of firing. Although the current used to administer bicuculline near this cell was half of that ejecting penicillin, the dilution of the bicuculline solution was such that approximately 2 nA (of the 60 nA) would have carried bicuculline cations from the micropipette. Hence the concentration of penicillin required to reduce the effect of a given amount of GABA was higher than that of bicuculline. Observations similar to these illustrated in Fig. 23 were made upon 8 Renshaw cells and 4 spinal interneurones (3 preparations).

Penicillin (30-100 nA) usually enhanced the firing rate of postcruciate neurones and reduced the inhibitory effect of GABA on all 9 cells tested (2 preparations). The inhibition of 2 of these neurones by glycine appeared to be as sensitive to antagonism by penicillin as that produced by GABA, but a full assessment of the specificity of the antibiotic as an antagonist of inhibitory amino acid receptors in the cortex was not made because of the relatively weak effects of glycine on these neurones (Curtis, Duggan, Felix, Johnston and McLennan, 1971).

An enhancement of the firing rates of cerebellar Purkinje cells was also observed with electrophoretic currents of penicillin (30-180 nA) and as observed in the spinal cord and postcruciate cortex, the inhibitory effect of GABA was reduced. Four Purkinje cells were tested (three preparations) and the firing rates of three of these cells were inhibited by local cerebellar surface

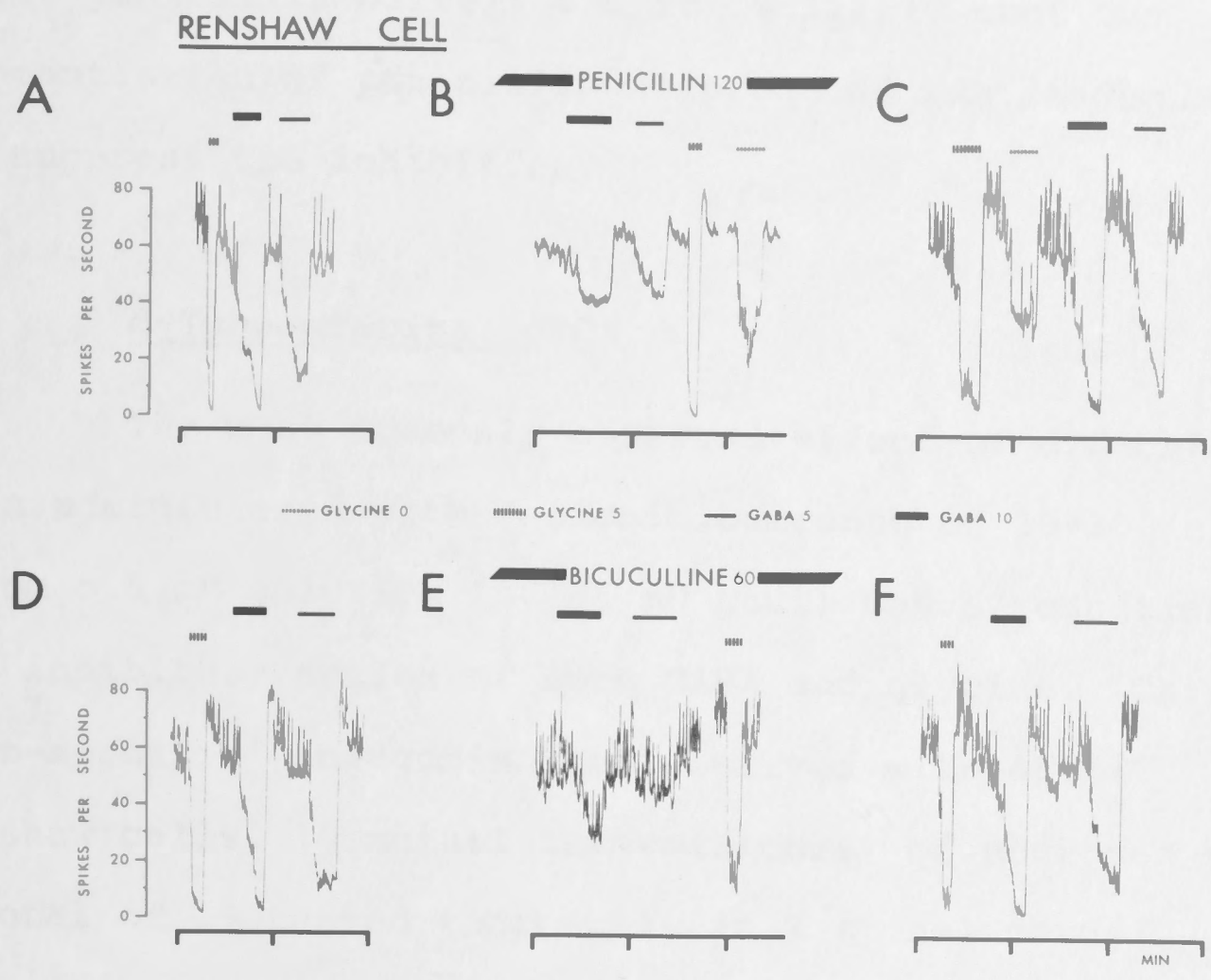


Fig. 23:

The effects of penicillin and bicuculline on the inhibition of the firing of a Renshaw cell by glycine and GABA. The electrophoretic administration of amino acids, penicillin and bicuculline is indicated by horizontal lines. The cell was excited by the continuous administration of DLH (10 nA) and was inhibited by glycine (dotted line, 0.5 nA) and GABA (solid line, 5, 10 nA). A, before; B, 2 min after beginning to eject penicillin with a current of 120 nA, the substance having been previously administered with 60 nA for 3 min; C, immediately after terminating this current; D, before; E, 1 min after beginning to eject bicuculline (60 nA); F, immediately after terminating this current.

stimulation. This inhibition was not affected by the maximum currents of penicillin that could be passed from three penicillin barrels and it is likely that the concentration of penicillin so produced was inadequate to suppress the inhibition.

(5) d-Tubocurarine (dTC)

The most commonly observed effect of dTC chloride when administered with cationic currents of 20-300 nA (from a 5 mM solution in 165 mM NaCl) was a reduction of the inhibitory action of both GABA and glycine. This 'non-specific' antagonism was observed with 48% (2 Renshaw cells, 10 spinal interneurones) of neurones from a total of 25 tested thoroughly in 9 spinal cord preparations. Tubocurarine had no effect on the inhibitory action of either GABA or glycine upon 32% (3 Renshaw cells, 5 interneurones) of the cells, whilst it antagonised only glycine on 12% (1 Renshaw cell, 2 interneurones) and only GABA on 8% (2 interneurones). In comparison, BMC (25-75 nA) specifically antagonised the inhibitory action of GABA on 14 of the 15 neurones (4 Renshaw cells, 10 interneurones) tested with both dTC and BMC. Strychnine also consistently and specifically antagonised the inhibitory action of glycine on all (2 Renshaw cells, 9 interneurones) neurones tested with both dTC and strychnine. These effects of BMC and strychnine were observed irrespective of the type of effect that dTC had on the actions of GABA and glycine. Almost all cells were excited by dTC and this effect appeared to be unrelated to the type of amino acid

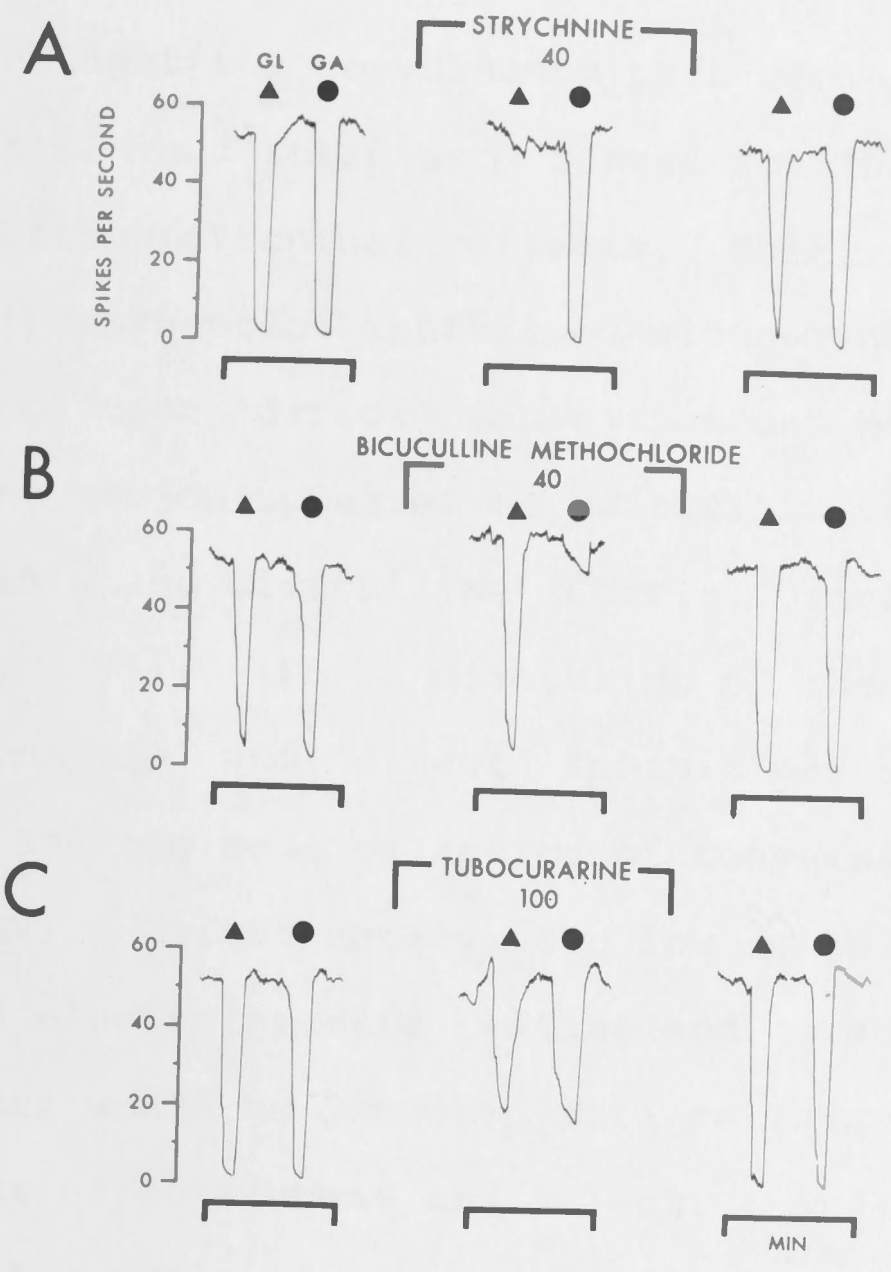


Fig. 24:

Maximal effects of strychnine (A), bicuculline methochloride (B) and (+)-tubocurarine (C) on the inhibition of firing of a spinal interneurone by glycine (GL, filled triangle = 9 sec. administration) and GABA (GA, filled circle = 13 sec. administration). Firing was maintained at a constant level with DLH (10-35 nA). From left to right: control responses, during administration of the indicated alkaloid, recovery. A, GL 15 nA, GA 20 nA, strychnine (2 mM in 165 mM NaCl) 40 nA for 2 min. Recovery at 5 min was incomplete. B, 6¼ min after A., GL 15 nA, GA 12.5 nA, bicuculline methochloride (BMC, 10 mM in 165 mM NaCl), 40 nA for 4 min. Recovery at 2 min. C, 2 min after B., GL 15 nA, GA 12.5 nA, bicuculline methochloride (BMC, 10 mM in 165 mM NaCl), 40 nA for 4 min. Recovery 2 min. C, 2 min after B., GL 15 nA, GA 12.5 nA, (+)-tubocurarine (5 mM in 165 mM NaCl) 100 nA for 3 min after 40 nA for 2 min., 60 nA for 3 min, 80 nA for 2 min. Recovery at 2 min.

DISCUSSION

Amongst the convulsants that were investigated, only shikimin and tutin were tested for their effects on the inhibition of spinal reflexes. Shikimin considerably reduced 'presynaptic' inhibition without having a significant effect upon 'direct' inhibition and hence its central convulsant action appeared to be similar to that of picrotoxinin and bicuculline (Curtis, Duggan, Felix and Johnston, 1971). (For a discussion of the significance of 'presynaptic' and 'direct' inhibitions of spinal reflexes and the mode of action of convulsants see Section IV). Unfortunately, the low solubility of shikimin prevented electrophoretic testing and more detailed experiments would be required at a cellular level to assess its effectiveness and selectivity as a GABA antagonist. Tutin, in low concentrations also reduced 'presynaptic' but not 'direct' inhibition, however, higher concentrations slightly reduced 'direct' inhibition. This finding, together with a reduction in the early phase of 'presynaptic' inhibitory curves (Fig. 19C), suggested that tutin might influence the inhibitory action of glycine as well as that of GABA. These two effects of tutin were readily apparent in the microelectrophoretic studies when increasing 'doses' of tutin antagonised both GABA and glycine. Thus the convulsant action of systemically administered tutin is due, at least in part, to antagonism of the inhibitory action of glycine and GABA. Although some selectivity was apparent for tutin as a GABA antagonist, this convulsant would be of little

use in microelectrophoretic studies of the nature of amino acid central inhibitory transmitters. Tutin has recently been reported to antagonise the GABA mediated increase in the muscle membrane conductance of the lobster (Homarus vulgaris) neuromuscular junction (Nistri, Constanti and Quilliam, 1974). In the lobster preparation tutin appeared to be approximately equipotent with picrotoxin; 1.5×10^{-6} - 6.0×10^{-6} M tutin or picrotoxin displaced the dose response curve to the right in a non-parallel manner.

Coriamyrtin may be more selective than tutin as a GABA antagonist, but it was impossible to investigate the effect of systemically administered coriamyrtin and to investigate thoroughly its effects at a cellular level. If there is a real difference in the degree of selectivity of these two compounds then it must be due to the secondary hydroxyl group which is absent from the structure of coriamyrtin, as this is the only difference between the two structures. This hydroxyl is cis to the lactone structure which is common to tutin, coriamyrtin, picrotoxin and dendrobine (Fig. 18) (but is absent from the shikimin molecule), and the close proximity of the lactone function and the hydroxyl group may make inter-conversion of the lactone from a five to a six membered ring possible. This interconversion thus could not occur in the coriamyrtin molecule. The other hydroxyl group, the bridgehead hydroxyl, is common to picrotoxinin, tutin, coriamyrtin and shikimin, but not to dendrobine, and it appears to be essential for the convulsant activity of those members of the series that have it in their structures

(Jarboe, Porter and Buckler, 1968). Neither the dendrobine nor the shikimin molecule has an oxirane ring, but from the structures of the remaining compounds of the series it seems that the presence and location of the oxirane ring are not important molecular features.

The explanation of the differences in pharmacological action between picrotoxinin and these related compounds is not readily apparent from their structures. Concentrations of dendrobine exceeding those required to suppress the inhibitory action of glycine were without effect on the action of GABA (Curtis, Duggan, Felix and Johnston, 1971; Curtis, Davies, Game, Johnston and McCulloch, 1973). Dendrobine, the only nitrogen containing member of this series can thus be considered as strychnine-like. Although relatively large intravenous doses of picrotoxinin (2 mg/kg, equivalent to 4 mg picrotoxin/kg) have not been found to modify spinal direct inhibition, the results of microelectrophoretic experiments on spinal interneurones have not provided uniquivocal evidence of a high degree of selectivity of this substance as a GABA antagonist (Curtis, Duggan, Felix, Johnston, 1971).

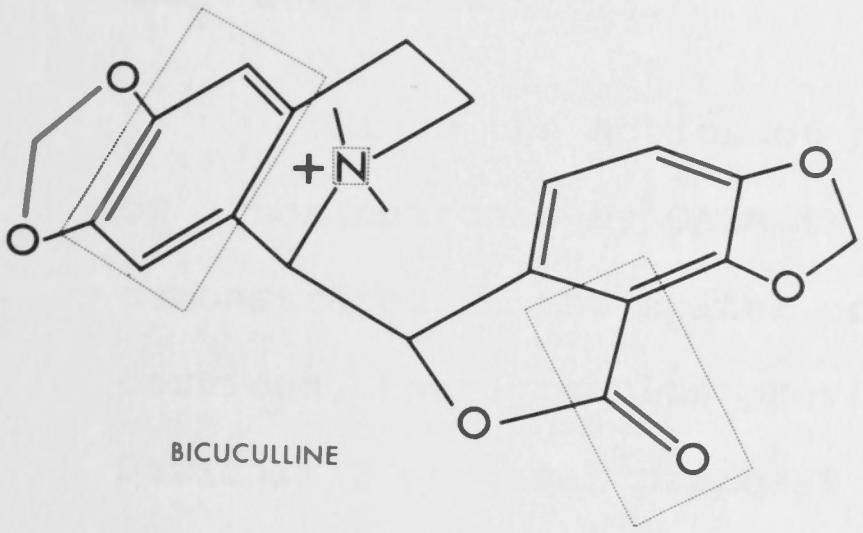
The reduction of dorsal root potentials and spinal prolonged (presynaptic) inhibition, but not short latency inhibition, by penicillin suggested that penicillin is an antagonist of the inhibitory action of GABA. The doses of penicillin that were required to modify spinal inhibitions were relatively high and may have been partly due to poor penetration of a blood-brain barrier (Fishman, 1966). Electrophoretic testing of penicillin

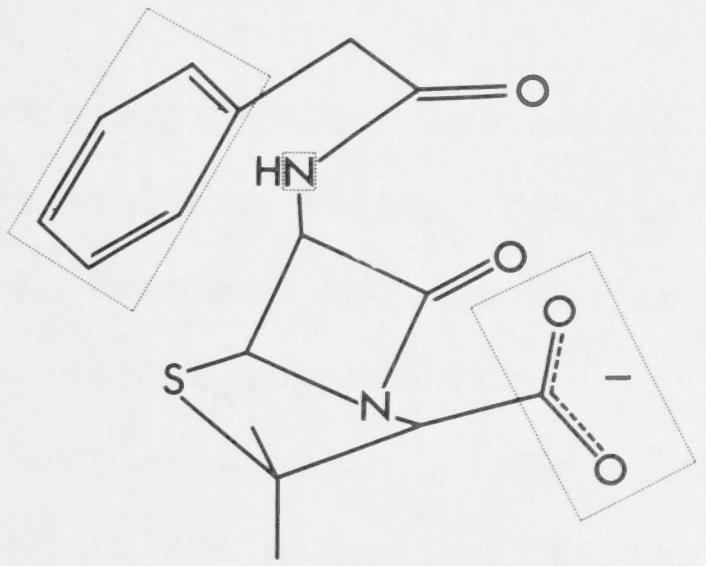
confirmed that it was a GABA antagonist. It was weaker, however, than bicuculline, a finding that was consistent with that of Davidoff who found it to be approximately 1/100 as effective as bicuculline in reducing 'presynaptic' inhibition and the depolarising action of GABA in the amphibian spinal cord (Davidoff, 1972a). Davidoff has recently extended these experiments with penicillin to the feline spinal cord preparation where the effects of penicillin included antagonism of the depressant action of GABA when electrophoretically administered near interneurones, and reduction of spinal prolonged inhibition (Davidoff, 1972b).

In the hermit crab (Eupagurus bernhardus) penicillin (1×10^{-4} - 1×10^{-3} M) has not been shown to antagonise the increase in membrane conductance induced by GABA at the neuromuscular junction, although lower concentrations of picrotoxin non-competitively antagonised this action of GABA (Earl and Large, 1973). Bicuculline, however, was reported to be either inactive or 40-50 times less potent than picrotoxin as an antagonist of GABA, or neurally evoked inhibition, in this preparation (Earle and Large, 1972, 1974), but in contrast bicuculline was more potent than picrotoxinin as a GABA antagonist at the stretch receptor of the crayfish (Eustacus armatus, McLennan, 1970). It thus seems likely that there is a species difference amongst crustacean GABA receptors and this possibility has been further considered by McLennan who has reported that bicuculline, bicuculline methochloride and picrotoxin were all approximately equipotent in concentrations of 0.5 - 13×10^{-7} M on stretch

receptor neurones of the crayfish Pacifastacus leniusculus (McLennan, 1973), a result that significantly differed from the earlier comparison in Eustacus armatus (McLennan, 1970). A difference was also found between the GABA receptors of the stretch receptor neurones and those of the neuromuscular junction of Pacifastacus leniusculus, for in the latter preparation the bicucullines were much less potent. Hence a useful comparison cannot be made between relative potencies of GABA antagonists when they are determined in different species of crustacea, similarly it is unlikely that results in crustacea can be extrapolated to the mammalian CNS.

Consideration of the structures of penicillin, bicuculline and GABA, when in the conformations depicted in Fig. 25, leads to the observation that in the structure of penicillin the benzyl grouping, the nitrogen atom on carbon 6 and the carboxylate anion can be in similar absolute positions to the equivalent groupings in bicuculline (shown by the 'boxed in' structures). When the benzyl groupings of penicillin and bicuculline and the carboxylate anion of penicillin and the C-C=O grouping of bicuculline are superimposed the nitrogen atoms of each molecule can be within approximately 0.8 Å of each other. (Other superimposable conformations are possible.) The similar relationship between bicuculline and certain conformations of some inhibitory substances whose actions are blocked by bicuculline (muscimol, imidazole acetic acid and 4-aminotetrolic acid: Beart, Curtis and Johnston, 1971; Curtis, Duggan, Felix and Johnston, 1971) have been previously discussed (Section I). Hence the structure-





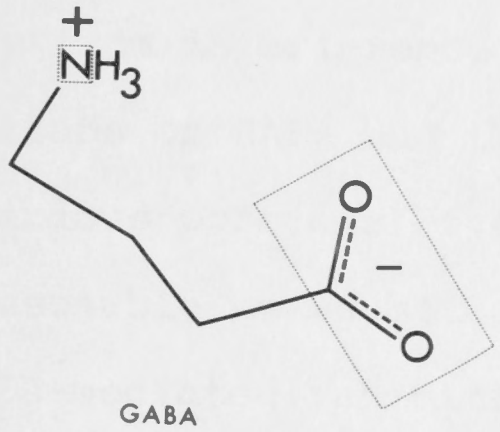


Fig. 25:

Conformations of bicuculline, GABA and benzyl penicillin showing structural similarities, drawn from Dreiding stereomodels.

activity relationships of penicillin support the hypothesis that GABA interacts with its receptor in a partially extended conformation, however penicillin, like bicuculline, does not significantly inhibit the uptake of GABA by cat spinal cord slices (Balcar and Johnston, 1973), this uptake being mediated by a receptor that appears to be structurally different from the postsynaptic GABA receptor.

Since the action of penicillin on the inhibition of other neurones by GABA is unlikely to differ from that demonstrated in the spinal cord, cerebral and cerebellar cortices, these results provide strong support for Davidoff's original proposal (Davidoff, 1972a). The acute convulsant effects of penicillin in the mammalian CNS may well arise mainly from interference with inhibitory transmitter functions of GABA and the differential sensitivity of various portions of the nervous system (Gloor, 1969) presumably stems both from the relative importance of GABA-mediated inhibitions in the functioning neural networks directly affected, and from their interconnections with other regions.

These experiments have clearly demonstrated that the convulsant or epileptogenic activities of tutin, coriamyrtin, shikimin, d-tubocurarine and penicillin may be due, at least in part to antagonism of the inhibitory amino acids, GABA and glycine. The comparison of the influence of dTC, BMC and strychnine on the inhibitory actions of GABA and glycine provided no support for the proposition that dTC is a useful GABA antagonist (Hill,

Simmonds and Straughan, 1973b). The usefulness of an antagonist of transmitter action at particular synapses depends both on its relative potency and the selectivity or specificity which it exhibits towards the effects of other substances and hence upon the degrees of confidence with which it can be used to aid transmitter identification at other sites. In this regard the utility of atropine and curare-like agents at peripheral muscarinic and nicotinic sites is well recognised, under conditions where excitation or inhibition of postsynaptic structures usually involves the activation of only one type of synapse. In contrast, most central neurones are influenced by a number of different transmitters and the tentative identification of a transmitter frequently depends upon a comparison between the effects of an antagonist of the appropriate synaptic process and its interaction with artificially administered compounds which mimic the action of the synaptically released transmitter. The basis for such tentative identification is thus selectivity of action of antagonist, and dTC would be of extremely dubious value in determining whether a glycine-like or GABA-like amino acid was the transmitter at central inhibitory synapses.

Amongst the convulsants that were investigated only coriamyrtin and penicillin were shown to selectively antagonise the inhibitory action of GABA. Coriamyrtin, however, is not readily available and penicillin is too weak to be of practical value as a GABA antagonist and its distribution after systemic administration is hindered by a blood-brain barrier.

VI. EXCITATION OF SPINAL NEURONES BY CONFORMATIONALLY RESTRICTED ANALOGUES OF L-GLUTAMIC ACID.

The relative flexibility of the L-glutamic acid molecule presents certain difficulties in determining the shape of the molecule during activation of receptors associated with excitation of central neurones. Van Gelder's proposal that L-glutamate interacts with its receptor in a folded conformation (Van Gelder, 1970) was discussed in the introduction to this thesis (Section I), and in an attempt to gain some insight into the possible shape(s) of glutamic acid during receptor interaction a study was made of four conformationally restricted analogues: (±)-cis-1-aminocyclohexane-1,3-dicarboxylic acid ('cycloglutamic acid'), ibotenic acid, kainic acid and its dihydro derivative.

Ibotenic acid, an isoxazole isolated from the mushroom Amanita muscaria (see Eugster, 1967) and kainic acid, a pyrrolidine derivative isolated from the seaweed Digenea simplex (Ueno, Nawa, Ueyanagi, Morimoto, Nakamori and Matsuoka, 1955; Murayama, Morimura, Nakamura and Sunagawa, 1965) have previously been shown to excite central neurones (Johnston, Curtis, de Groat and Duggan, 1968; Shinozaki and Konishi, 1970). 'Cycloglutamic acid' is an inhibitor of glutamine synthetase (Gass and Meister, 1970) and thus presumably interacts with an active site on this enzyme capable of interacting with glutamate. In each of these cyclic compounds the ring restricts rotation about bonds equivalent to the C_α-C_β and/or C_β-C_γ in L-glutamic acid.

RESULTS

The approximate relative potencies (Table 5) of the amino acids when electrophoretically ejected with anionic currents were assessed on the basis of results obtained with 22 interneurones and 15 Renshaw cells (10 spinal cord preparations) using the following solutions: 'cyclo-glutamic' acid (I M, adjusted to pH 7.3 with NaOH), dihydrokainic acid (0.2 M, pH 7), L-glutamic acid (0.5 M, pH 8), DL-homocysteic acid (0.2 M, pH 7.5), ibotenic acid (0.2 M, pH 7), kainic acid (0.1 M, pH 8) and N-methyl-D-aspartic acid (0.1 M, pH 8).

TABLE 5 Approximate potency ranges of excitant amino acids relative to L-glutamic acid, based on the ejecting currents required to achieve equal increases in firing frequency of spinal neurones.

Amino acid	Potency range relative to that of L-glutamic acid; number of neurones tested in brackets	
Kainic	8 - 80	(19)
N-Methyl-D-aspartic	7 - 20	(10)
DL-Homocysteic	3 - 7	(12)
Ibotenic	2 - 6	(6)
L-Glutamic	1	(35)
'Cycloglutamic'	0.7 - 0.8	(3)
Dihydrokainic	0.06 - 0.6	(5)

Kainic acid was more potent than N-methyl-D-aspartic acid, previously the most potent amino acid excitant reported (Curtis and Watkins, 1963), but dihydrokainic acid was the weakest excitant of the series. Fig. 26 illustrates the effect of kainic acid on the firing rate of an interneurone in comparison with that of L-glutamic, DL-homocysteic and N-methyl-D-aspartic acids. The actions of kainic and N-methyl-D-aspartic acids were relatively slow in both onset and offset, compared with that of L-glutamic acid; this was also apparent with 'cyclo-glutamic', dihydrokainic and ibotenic acids.

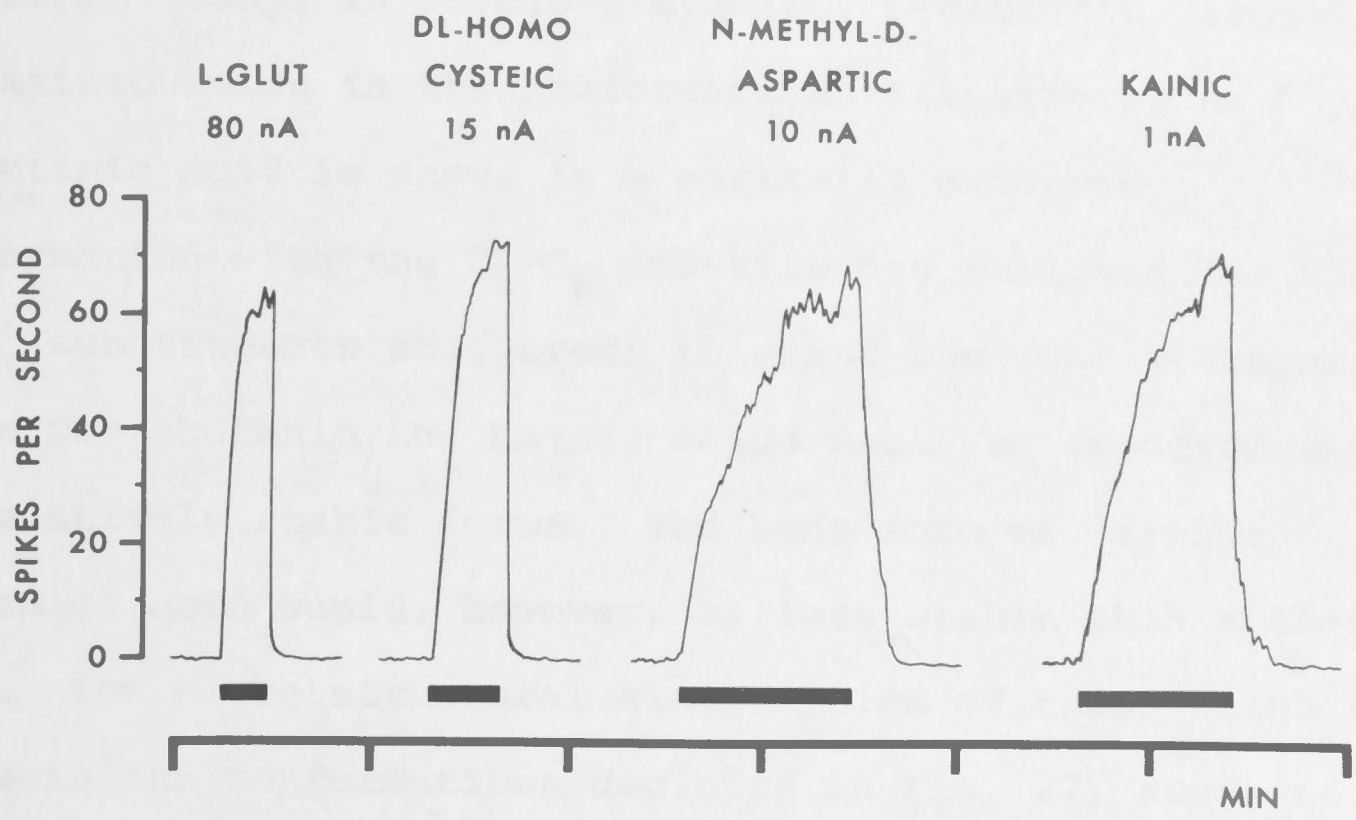


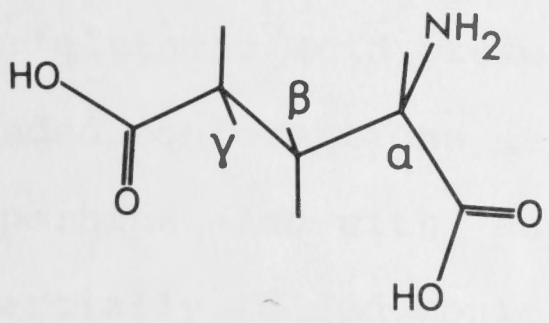
Fig. 26:

Comparisons of the excitatory actions of L-glutamic (L-GLUT, 80 nA), DL-homocysteic (15 nA), N-methyl-D-aspartic (10 nA) and kainic (1 nA) acids on a spinal interneurone. The horizontal bars indicate periods of electrophoretic ejection. Ordinate: firing frequency in spikes per second. Abscissa: time in minutes.

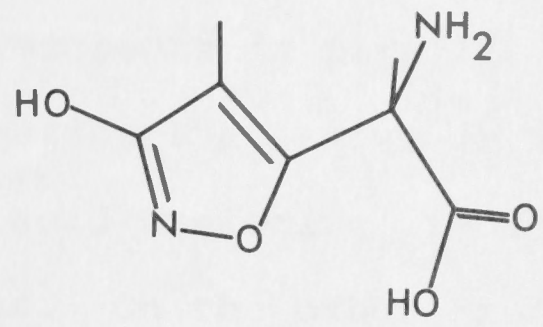
DISCUSSION

An analysis of molecular models indicates that there is a unique juxtaposition of the equivalent ionizable groups in 'cyclo-glutamic', L-glutamic, ibotenic and kainic acids in the conformations illustrated in Fig. 27. L-glutamic acid is shown in a partially extended conformation with the C_α-C_β substituents eclipsed and the C_β-C_γ substituents staggered; this and the conformations shown for ibotenic and kainic acids would be expected to be relatively stable forms. The boat form of 'cyclo-glutamic' acid would, however, be less stable than a chair form. The close structural similarities of these amino acids in the conformations depicted in Fig. 27, suggests that they all interact with the same receptor and that L-glutamate interacts with the receptor in a partially extended conformation. Tests with a specific glutamate antagonist, when one becomes available, may be able to confirm that these compounds interact with a common receptor.

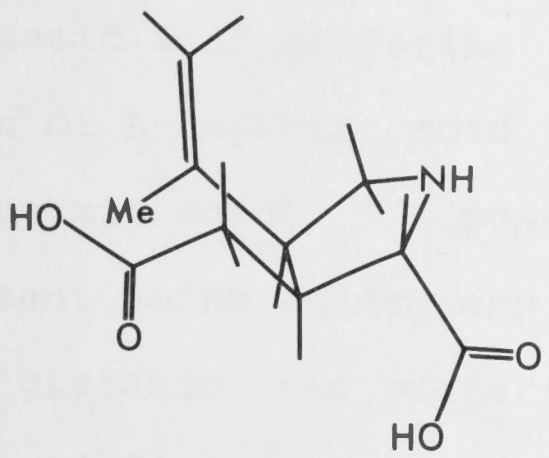
The observation that dihydrokainic acid was much less potent than kainic acid, indicates that the double bond in the kainic acid molecule is an important feature in determining the potency of the molecule as an excitant. This double bond, the conformation of which is arbitrarily illustrated in Fig. 27, might bind to a specific site on the receptor or might influence the preferred conformation and the electronic properties of the neighbouring carboxyl group. Alternatively, the double bond might only influence the electronic properties of the carboxyl group.



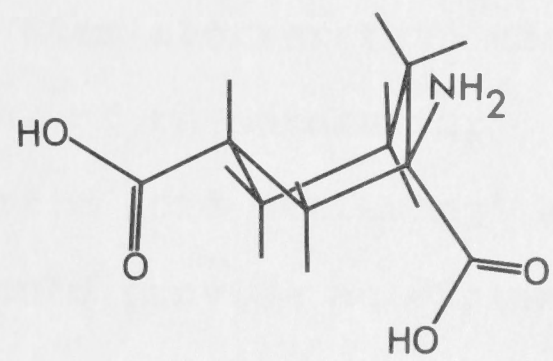
GLUTAMIC ACID



IBOTENIC ACID



KAINIC ACID



"CYCLO-GLUTAMIC" ACID

Fig. 27:

Conformations of glutamic, ibotenic, kainic and 'cycloglutamic' acids in which the ionisable groups occupy the same absolute positions.

The ionizable groups of L-aspartic acid and NMDA acid cannot match those illustrated in Fig. 27 for L-glutamic acid and its analogues, hence it is possible that excitation by L-glutamic acid and L-aspartic acids results from preferential interaction by these two amino acids with different receptors. L-glutamic acid might interact with 'glutamic acid prefering' receptors in partially extended conformations (approximating that shown in Fig. 27), and perhaps also with 'aspartic acid prefering' receptors in partially folded conformations. On the other hand, L-aspartic acid may interact only poorly, if at all, with 'glutamic acid prefering' receptors since the carbon chain of L-aspartic acid is one atom shorter than that of L-glutamic acid. Two populations of receptors for excitant amino acids, one 'aspartic acid prefering' and one 'glutamic acid prefering' would provide an explanation of the differences in the relative potency of L-glutamic and L-aspartic acids as excitants of different central neurones. For example, the differential sensitivity of Renshaw cells and dorsal horn interneurones to L-glutamate and L-aspartate (Duggan, 1974) may be due to different populations of receptors for these excitant amino acids (see Section VII).

VII. THE DIFFERENTIAL SENSITIVITY OF SPINAL INTERNEURONES AND RENSHAW CELLS TO KAINATE AND N-METHYL-D-ASPARTATE (NMDA)

Duggan found that Renshaw cells, which have not been shown to be monosynaptically excited by impulses in primary afferent fibres (Curtis and Ryall, 1966b; Ryall and Piercey, 1971) are more sensitive to electrophoretically administered L-aspartate than to L-glutamate, whereas spinal dorsal horn interneurones that are monosynaptically activated from the periphery are more sensitive to L-glutamate than to L-aspartate (Duggan, 1974). The problems associated with assessing the relative sensitivity of particular neurones to electrophoretically administered compounds were discussed in the introduction to this thesis (Section I), but despite these difficulties the differential sensitivity of Renshaw cells and spinal dorsal horn interneurones supports the hypothesis that L-glutamate is an excitatory transmitter released by primary afferent fibres and L-aspartate an excitatory transmitter released by excitatory spinal interneurones (Graham, Shank, Werman and Aprison, 1967). This hypothesis was previously based on neurochemical evidence of the spinal distribution of these excitant amino acids. High levels of L-glutamate occur in dorsal roots (Graham, Shank, Werman and Aprison, 1967) and dorsal root ganglia (Duggan and Johnston, 1970) whilst within the cord the highest levels are found in dorsal grey matter (Graham, Shank, Werman and Aprison, 1967). In contrast, L-aspartate levels are highest in the ventral grey. Furthermore, the number of interneurones in

the central region of the spinal cord can be reduced by temporary aortic occlusion and the resulting inter-neuronal loss has been correlated with the subsequent decrease in aspartate levels (Davidoff, Graham, Shank, Werman and Aprison, 1967).

Although Renshaw cells were reported to be consistently more sensitive to L-aspartate than to L-glutamate, the mean glutamate:aspartate 'potency ratio' was only 1.2 whilst the mean 'potency ratio' for inter-neurones was only 0.8, where the 'potency ratio' was an equipotent current ratio , the ratio of ejecting currents which produced equal and submaximal responses (Duggan, 1974). The molecules of glutamate and aspartate are flexible and the relatively small potency difference may result from glutamate interacting with both glutamate and aspartate receptors. The structure-activity relationships of kainic, ibotenic and 'cycloglutamic' acids, as discussed in the previous section (Section VI), suggest that glutamate might interact with the glutamate receptor in a partially extended conformation and perhaps also with the aspartate receptor in a partially folded conformation. Aspartate, however, has one less carbon atom in its chain and thus probably cannot interact with such a glutamate receptor. If L-glutamate is 'non-specific', in that it interacts with both glutamate and aspartate receptors, then a greater relative difference in the sensitivities of Renshaw cells and interneurones would be expected with glutamic acid analogues that were more selective for the glutamate receptor.

Due to its restricted conformation it is unlikely that kainic acid could occupy aspartate receptors and hence should be more selective than glutamate in terms of interacting with glutamate, but not with aspartate receptors. NMDA which is comparible in potency to kainate is probably too small a molecule to interact with the glutamate receptor and hence an investigation was made of the relative sensitivities of dorsal horn interneurones and Renshaw cells to kainate and NMDA.

RESULTS

Seventeen Renshaw cells and 21 spinal interneurones activated with a short central latency (<2 msec) from peripheral nerves were tested in 7 cats using 7 micropipettes. Responses to kainate (5 mM in 250 mM NaCl, pH adjusted to 8.3 with NaOH) and NMDA (50 mM in 200 mM NaCl, pH 8.3) were generally slower in onset and recovery than those to glutamate or aspartate (Fig. 28).

TABLE 6 Sensitivity ratios of spinal interneurones and Renshaw cells to kainate, relative to NMDA

Cell type	Classes of values of sensitivity ratios		
	>10	10	<10
Interneurone	16	4	1
Renshaw	0	0	17

The table contains numbers of cells with values of sensitivity ratio in classes.

A highly significant difference ($P<0.001$ for the Chi-squared statistic of the contingency table, and $P<0.001$ for Student's t statistic for the means and standard errors) was found in the relative sensitivities of the two groups of neurones (Table 6). Fig. 28 illustrates a comparison of the sensitivity of a Renshaw cell and an interneurone to the excitants electrophoretically ejected from the same micropipette. The sensitivity ratio

(K:NMDA) for the interneurone was 16 and for the Renshaw cell it was 2.5; all of the Renshaw cells that were tested had sensitivity ratios that were less than 10, whilst only one of the sample of 21 interneurones had a ratio that was less than 10. The mean and standard error of the sensitivity ratios were 17.8 $\pm$ 2.2 for interneurones and 4.3 $\pm$ 0.5 for Renshaw cells, there being a 4-5 fold difference in the sensitivity ratios for the two groups of neurones. Thus, with sensitivity to NMDA as the basis of comparison, interneurones were relatively more sensitive to kainate than were Renshaw cells.

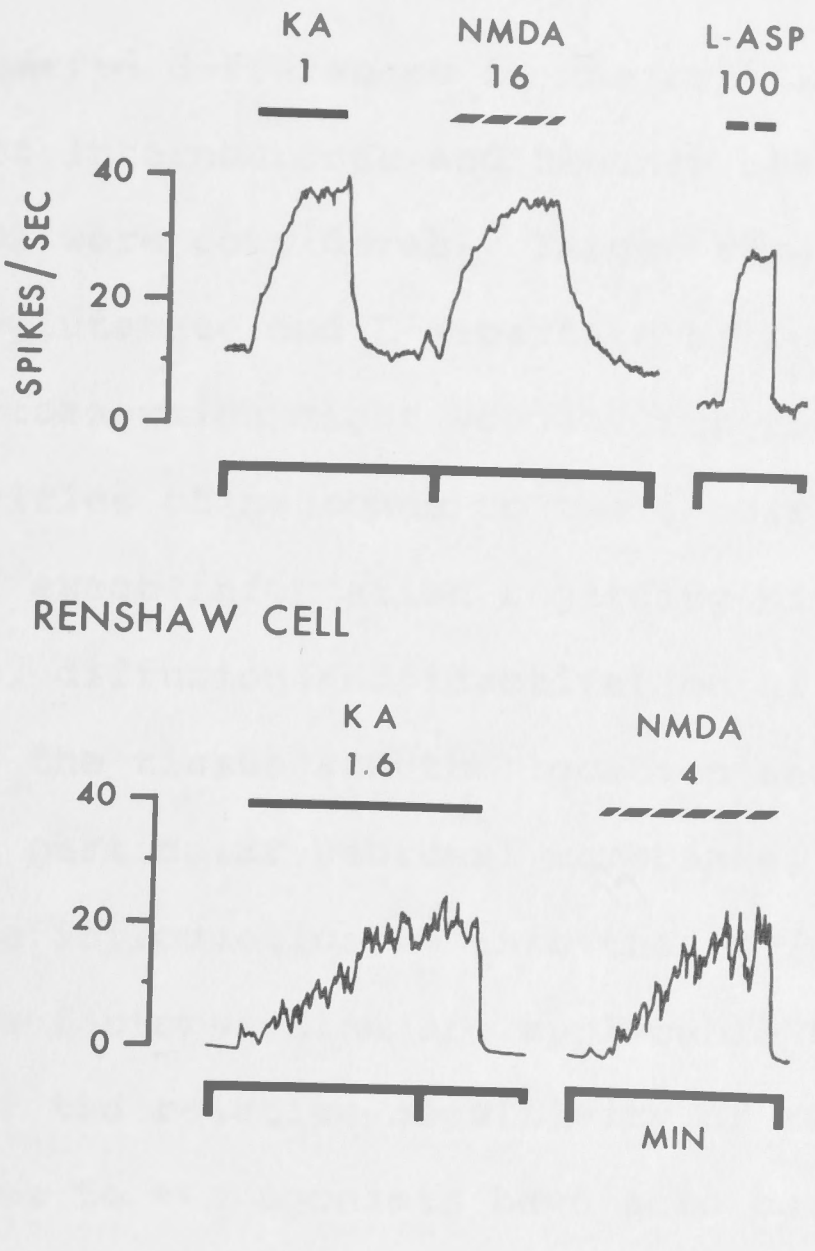


Fig. 28:

Comparisons of the relative sensitivities of a spinal interneurone and a Renshaw cell to electrophoretically administered kainate (KA) and N-methyl-D-aspartate (NMDA). The horizontal bars indicate periods of ejection and the currents shown have been corrected for dilution in NaCl (see text): Kainate = 5 mM in 250 mM NaCl and NMDA = 50 mM in 200 mM NaCl. The sensitivity ratio (KA:NMDA) of the interneurone was 16 and that of the Renshaw cell was 2.5. A typical response to L-aspartate (L-ASP, 100 nA) is included for comparison with the responses to kainate and NMDA. Ordinates: firing frequency in spikes per second. Abscissa: time in minutes.

DISCUSSION

The observed differences in the relative sensitivities of interneurones and Renshaw cells to kainate and NMDA were considerably larger than those reported for L-glutamate and L-aspartate by Duggan (1974). The various factors which might account for the differences in the sensitivities of neurones to two agonists, factors such as lack of exact information regarding micropipette characteristics, diffusion and inactivation of ejected agonists within the tissue and the location and specificity of receptors on particular neuronal membranes, have been discussed in the introduction to this thesis (Section I). In addition, the factors which are applicable to the determination of the relative sensitivity of two particular types of neurones to two agonists have also been discussed in general terms, and they will now be related to the results of the present investigations.

The microelectrophoretic technique does not enable a measurement to be made of the relative intrinsic activity or efficacy of kainate and NMDA at neuronal receptors. In addition, kainate and NMDA are probably distributed differently in the tissue from glutamate and aspartate during electrophoretic administration. L-glutamate and L-aspartate are taken up by high affinity transport systems in slices of spinal cord in vitro, and NMDA appears not to be a substrate for these systems (Balcar and Johnston, 1972b). The slow recovery of kainate and NMDA responses suggests that active uptake of these excitants

contributes very little to the termination of their responses, although to some extent these slow time courses may reflect slow dissociation of amino acid-receptor complexes. Kainate is a weak competitive inhibitor of glutamate high affinity uptake by brain tissue slices (see McCulloch, Johnston, Game and Curtis, 1974) and thus can only be a relatively poor substrate for this transport system. It is possible that both kainate and NMDA may be taken up by other transport processes, and this possibility needs to be investigated using radioactively labelled compounds. To allow a full discussion of the present results, however, it would ultimately be necessary to compare the neurochemical environment of the Renshaw cell with that of the dorsal horn interneurone.

The neurochemical environment of Renshaw cells and interneurones determines, in part, the tissue volume that is affected by electrophoretically ejected kainate and NMDA. The relative sensitivity of these two types of neurones would depend not only on the relative distribution volumes of ejected kainate and NMDA, but also on the distribution patterns of the excitatory receptors. At present the location of amino acid excitatory synapses on Renshaw cells and interneurones is not known, but it seems likely from studies in other regions of the central nervous system that they are located predominantly on the dendrites (see Eccles, 1964).

If it is assumed that kainate is more selective for the glutamate receptor than is glutamate, and that

NMDA is too small a molecule to interact with the glutamate receptor, then the present results are consistent with the presence of both glutamate and aspartate receptors on spinal neurones, there being fewer glutamate receptors, in comparison with aspartate receptors, on Renshaw cells than on interneurones. Hence, if the excitatory amino acid receptors are located on postsynaptic membranes (see Curtis, Duggan, Felix, Johnston, Tebēcis and Watkins, 1972) then the results also provide additional support for the proposition that glutamate is the excitatory transmitter at primary afferent synapses in the spinal cord and that aspartate is the excitatory transmitter released by spinal excitatory interneurones. By using amino acid analogues to reveal such significant differences in the amino acid sensitivity of particular populations of neurones it may be possible to distinguish glutamate-mediated excitatory pathways from those mediated by aspartate in other regions of the central nervous system, although the use of specific antagonists of the excitants will be required to fully assess the synaptic role of these amino acids. A study of the interactions between such antagonists and kainate and NMDA may also provide an indication of the degree of specificity of all these compounds in relation to amino acid receptors at excitatory synapses.

VIII. POTENTIAL ANTAGONISTS OF THE POST-SYNAPTIC ACTION OF L-GLUTAMIC ACID

Specific post-synaptic antagonists of glutamate or aspartate would be of considerable use in assessing the transmitter roles of these two excitant amino acids and in studying the nature of the receptors that are involved. For these reasons many compounds have been tested electrophoretically as potential glutamate or aspartate antagonists (see, for example, Curtis, Duggan, Felix, Johnston, Tebēcis and Watkins, 1972; Haldeman and McLennan, 1972; Davies and Watkins, 1973), but few have shown a sufficient degree of selectivity towards amino acid excitation to unequivocally distinguish amino acid from cholinergic synaptic excitation, and no antagonist has yet been shown to unequivocally distinguish between aspartic and glutamic acid excitation.

Lysergic acid diethylamide has been reported to block the excitation of neurones in the feline brain stem, but only those neurones that were excited by 5-hydroxytryptamine were susceptible to antagonism of glutamate excitation by lysergic acid diethylamide (Boakes, Bradley, Briggs and Dray, 1970). Although lysergic acid diethylamide antagonised the excitatory action of L-glutamate without antagonising the excitatory response to acetylcholine or DLH, it also antagonised the excitatory action of 5-hydroxytryptamine and its effect on L-aspartic excitation was not tested. Of the glutamic acid derivatives that have been tested as potential

glutamate antagonists, α-methyl-DL-glutamate and L-glutamic acid diethyl ester (GDEE), which themselves possess little or no excitatory activity, were reported to be more effective in reversibly reducing the excitatory action of L-glutamate than those of acetylcholine, L-aspartate or DLH on neurones of the feline thalamic nuclei (Haldeman, Huffman, Marshall and McLennan, 1972). The synaptic excitation of cells in the nucleus ventralis posterolateralis, evoked by electrical stimulation of hind limb afferent nerves, was also reduced by GDEE, although blockade of synaptic activation was not necessarily observed on every occasion when the response to electrophoretically administered L-glutamate was abolished. The relative selectivity of L-glutamate antagonism by GDEE was further demonstrated on the amino acid excitation of neurones in the feline cuneate nucleus and spinal cord, and in addition the reduction in the sensitivity of cuneate neurones and spinal neurones to L-glutamate by GDEE was accompanied by reduced synaptic excitation by impulses in the dorsal columns and peripheral nerves respectively (Haldeman and McLennan, 1972). Stone (1973) has also reported that GDEE both reduces the glutamate induced excitation of pyramidal tract interneurones in the rat cerebral cortex more than acetylcholine induced excitation, and blocks synaptically evoked spikes resulting from pyramidal tract stimulation. Another study of α-methyl-glutamate and GDEE failed to demonstrate selective antagonism of the excitation of feline spinal interneurones, Renshaw cells, ventrobasal thalamic neurones, lateral

geniculate neurones and pyramidal tract neurones by L-glutamate, L-aspartate and DLH (Curtis, Duggan, Felix, Johnston, Tebēcis and Watkins, 1972). Steinberg, Altman and ten Bruggencate (1974) found no consistent antagonism by GDEE of the excitation of feline red nucleus neurones by L-aspartate, DLH and L-glutamate, although DLH and L-glutamate excitation were apparently reduced. These experiments in the red nucleus indicated a tendency for GDEE to hyperpolarise cell membranes and a further report (Zieglegansberger and Puil, 1973b) that both α-methyl-glutamate and GDEE hyperpolarise spinal neurones and increase membrane conductance suggests that the reduction of amino acid excitation is not completely due to selective antagonism at neuronal membrane receptors. Similarly, it is unlikely that proline which has been reported to antagonise the depolarisation of retinal neurones by L-glutamate (Van Harreveld and Fifkova, 1973) will prove to be a useful glutamate antagonist for it is a glycine-like amino acid (see Curtis and Johnston, (1974a; Young and Snyder, 1973) and consequently may alter the conductance of retinal neuronal membranes.

Aporphines have been tested for antiglutamate activity, and although nuciferine (1-5,6-dimethoxyaporphine) when administered electrophoretically blocked the excitant action of both acetylcholine and L-glutamate on Renshaw cells of the rat (Duggan, Lodge, Biscoe and Headley, 1973), and apomorphine diminished the sensitivity of feline Renshaw cells to acetylcholine, L-glutamate and L-aspartate (Curtis, Duggan, Felix, Johnston, Tebēcis and Watkins, 1972), 2-methoxyaporphine did not affect acetylcholine

excitation of feline Renshaw cells, ventrobasal thalamocortical neurones or pyramidal tract neurones, but it simultaneously reduced the effects of L-aspartate, L-glutamate and DLH. In this same study Curtis and coworkers found that L-methionine-DL-sulphoximine (MSO) reduced the excitatory effects of L-aspartate, L-glutamate and DLH, but did not affect acetylcholine excitation, of neurones in the feline spinal cord, ventrobasal thalamus, lateral geniculate nucleus and cerebral cortex. Similar results were obtained using DL-methionine-DL-sulphoximine (Haldeman and McLennan, 1972), however the low solubility of MSO and its inability to distinguish between L-aspartic and L-glutamic acid excitation detracts from its usefulness.

Due to the limited ability of the presently available glutamate antagonists to specifically antagonise glutamate at the post-synaptic receptor, investigations continue to be made to find a more specific glutamate antagonist. From glutamate structure-activity relationship studies it would seem necessary for a glutamate antagonist molecule to have a basic group plus one or two electronegative groups in place of the fully anionic groups of the agonist molecules. On this basis a number of compounds (Table 7) have been tested for glutamate antagonism, which have a basic group and oxygen atoms (or electronegative groups) situated little differently from where they are located in the extended forms of glutamate and aspartate (see Sections I and VI). In addition, a number of compounds (Table 7) have been tested which have a structural resemblance to glutamate and have central

actions which may be due to glutamate antagonism (for example, the action of a CNS depressant or a 'central muscle relaxant'). A study has also been made of the effects of 1-hydroxy-3-amino-pyrrolidone-2 (HAP) on central neurones. When administered electrophoretically HAP has been reported to reduce the sensitivity of neurones in the cerebral cortex of the cat to L-glutamate and L-aspartate with little or no influence on either the spontaneous firing rate, the sensitivity to acetylcholine, or the amplitude of extracellularly recorded action potentials (Davies and Watkins, 1973). Systemically administered HAP produces a variety of centrally mediated depressant effects in a number of species, including depression of spinal polysynaptic reflexes (Bonta, De Vos, Grisjsen, Hillen, Noach and Sim, 1971), and hence the effect of HAP on spinal neurones was investigated.

RESULTS AND DISCUSSION

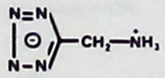
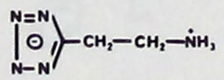
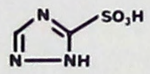
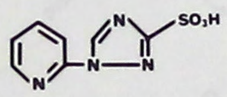
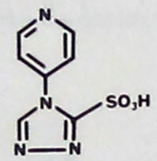
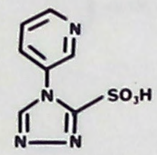
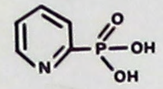
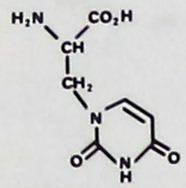
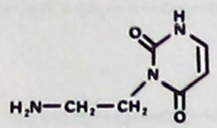
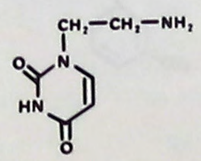
(1) Compounds of Table 7

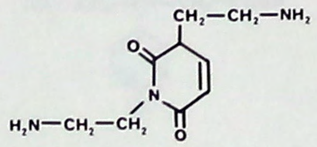
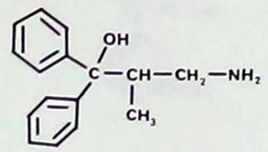
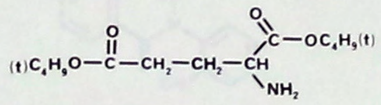
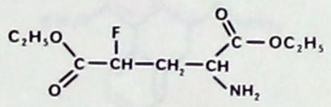
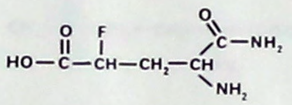
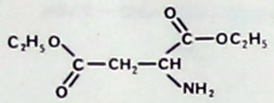
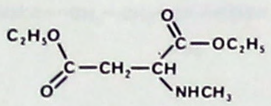
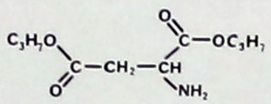
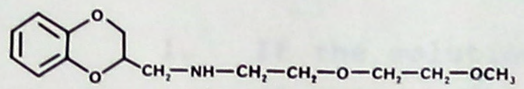
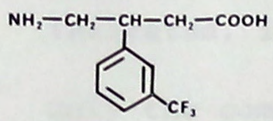
The compounds listed in Table 7 were tested as potential glutamate antagonists on spinal interneurones and Renshaw cells. A total of 11 spinal cord preparations were used and apart from those compounds which were either weak excitants (compounds V, VI and VIII) or had no detectable effect (compounds III, IX, XV, XVI and XXIII), they were all found to be depressants and unsuitable as specific glutamate antagonists. The compounds were ejected with either cationic or anionic currents (20-100 nA), depending upon the predominant species present in the solution used (see Table 7), except for compound XXV which was tested with both cationic and anionic currents.

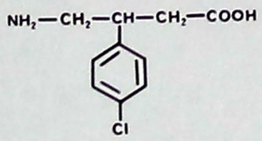
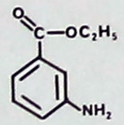
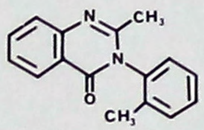
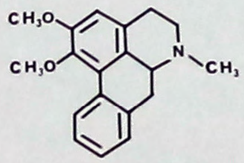
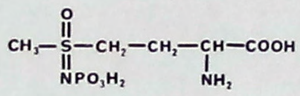
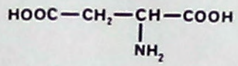
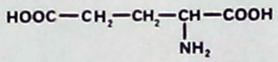
(a) Compounds I and II (tetrazole derivatives)

Compounds I and II were weak depressants (approximately 1/8 and 1/4 of the potency of GABA respectively) of spinal neurones. Fig. 29 illustrates the use of a Renshaw cell to test a potential glutamate antagonist, in this case compound II: compound II was ejected by a cationic current of 25 nA and this reversibly reduced the excitant effect of electrophoretic pulses of both acetylcholine (15 nA) and DLH (20 nA), this non-specific depressant effect becoming more pronounced with continuing administration of compound II. Although the neurone depicted in this figure fired at a low spontaneous rate, other neurones

TABLE 7 COMPOUNDS TESTED FOR ANTAGONISM OF L-GLUTAMIC ACID EXCITATION

No.	COMPOUND	FORMULA	SOLUTION Molarity	SOLUTION pH	No. NEURONES	CURRENT (nA)	ANTAGONISM	
I	5-Aminomethyltetrazole		0.2	3 (HCl)	3	(+) 20-90	LG LA ACh DLH	A weak neuronal depressant
II	5-Aminoethyltetrazole		0.2	5 HBr	6	(+) 20-50	LG LA ACh DLH	A weak neuronal depressant
III	1,2,4-Triazole-3-sulphonic acid		0.2	5 (NaOH)	4	(-) 30-50		No effect detected.
IV	1(2-Pyridyl)-1,2,4-triazole-3-sulphonic acid		0.2	8 (NaOH)	3	(-) 20-80	LG LA	Weak excitation occasionally observed.
V	4-(4-Pyridyl)-1,2,4-triazole-3-sulphonic acid		0.2	8 (NaOH)	9	(-) 25-100		
VI	4-(3-Pyridyl)-1,2,4-triazole-3-sulphonic acid		0.2	8 (NaOH)	3	(-) 40-50	LG LA	
VII	Pyridine-2-phosphonic acid		0.2	8 (NaOH)	3	(-) 20-60	LG LA	
VIII	DL-Willardine		0.2	3 (HCl)	3	(+) 20-40		Weak excitation observed.
IX	3-(2-Aminoethyl) uracil		0.2	4 (HCl)	3	(+) 20-50		No effect detected.
X	1-(2-Aminoethyl) uracil		0.2	4 (HCl)	7	(+) 30-80	LG LA	

No.	COMPOUND	FORMULA	SOLUTION Molarity	SOLUTION pH	No. NEURONES	CURRENT (nA)	ANTAGONISM	REMARKS
XI	1,3-Di-(2-aminoethyl) uracil		0.2	4 di-HCl	5	(+) 40-80	LG LA	Spike amplitude reduced.
XII	DL-α-(2-Amino-1-methyl-ethyl benzhydrol		0.2	5 HCl	7	(+) 20-50	LG	Spike amplitude reduced.
XIII	L-Glutamic acid di-tert-butyl ester		0.2	5 HCl	9	(+) 10-60	LG LA ACh	Spike amplitude reduced.
XIV	4-Fluoroglutamic acid diethyl ester		0.2	5 HCl (NaOH)	3	(+) 20-60	LG LA ACh	
XV	4-Fluoroglutamic acid-1-amide		0.2	3 HCl (NaOH)	5	(+) 30-60		No effect detected.
XVI	DL-Aspartic acid diethyl ester		0.2	3 HCl	8	(+) 20-50		No effect detected.
XVII	N-Methyl-DL-aspartic acid diethyl ester		0.2	4 HBr	3	(+) 30-60	LG ACh	Spontaneous rate depressed.
XVIII	N-Methyl-DL-aspartic acid di-n-propyl ester		0.2	4 (HCl.)	3	(+) 35-60	LA ACh	
XIX	Ambenoxan		0.2	5 HCl	4	(+) 20-50	LG LA ACh	Spike amplitude reduced. Spontaneous rate depressed.
XX	β-(m-Trifluoromethyl-phenyl)-γ-aminobutyric acid		0.2	3 (HCl)	4	(+) 30-50	LG LA	

No.	COMPOUND	FORMULA	SOLUTION Molarity	pH	No. NEURONES	CURRENT (nA)	ANTAGONISM	REMARKS
XXI	β-(p-Chlorophenyl)-γ-aminobutyric acid (βCPG)		0.2	3	4	(+) 30-60	LG LA ACh	
XXII	m-Aminobenzoic acid ethyl ester methanesulphonate (metacaine)		0.020*	3 MS	4	(+) 30-100	LG LA ACh	Spike amplitude reduced.
XXIII	2-Methyl-3-(o-tolyl)-4(3H)-quinazolinone		0.025*	3 HCl	5	(+) 20-50		No effect detected.
XXIV	1-5,6-Dimethoxyaporphine (nuciferine)		0.050*	3 HCl	4	(+) 30-50	LG ACh	Spike amplitude reduced.
XXV	L-Methionine-D,L-sulphoximine phosphate		0.2	8	10	(-) 10-90 (+) 30-80		No effect detected or spike amplitude reduced. Weak excitation observed, but inconsistent.
L-Aspartic acid			1	8 (NaOH)				
L-Glutamic acid			1	8 (NaOH)				

*in 0.165 M NaCl

LG = L-glutamic acid
LA = L-aspartic acid
ACh = acetylcholine

Notes to table 7

1. If the solution of a compound was prepared from a salt, the type of salt used is indicated after the pH of the solution. If the pH of the solution was adjusted the added acid or alkali is indicated, in brackets, in the pH column.

2. Only the compounds which are listed in the table as antagonising the excitant action of acetylcholine were tested on Renshaw cells.

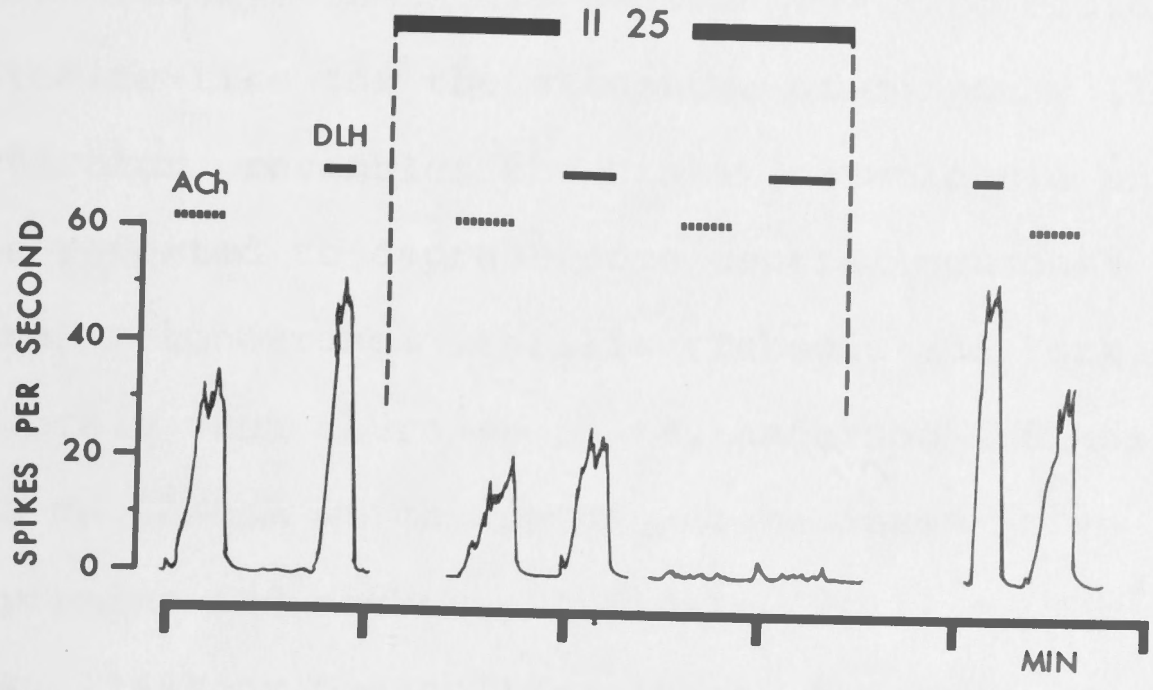


Fig. 29:

Effect of compound II (5-aminoethyltetrazole) on the chemical excitation of a Renshaw cell. The cell was excited by alternate electrophoretic pulses of acetylcholine (ACh, 15 nA) and DL-homocysteate (DLH, 20 nA), indicated by the broken and solid horizontal lines, respectively. Compound II (25 nA) was administered for the period indicated by the solid horizontal bar and broken vertical lines. Ordinate: firing frequency in spikes per second. Abscissa: time in minutes.

were tested in which it was possible to demonstrate a reduction in spontaneous rate during the continuous administration of either compound I or compound II. If the tetrazole ring is electronegative, compound I (5-amino-methyltetrazole) could be glycine-like and compound II (5-aminoethyltetrazole) GABA-like. Alternatively, these two weak depressants could be histamine-like for the structure of compound II, in particular, resembles the histamine molecule which has been reported to depress some central neurones including spinal motoneurones (Phillis, Tebēcis and York, 1968) and brain stem neurones (Haas, Anderson and Hösli, 1972) by a mechanism which appears to be insensitive to strychnine and either picrotoxin (Phillis, Tebēcis and York, 1968) or bicuculline (Haas, Anderson and Hosli, 1972).

(b) Compounds III - VII (triazole derivatives and pyridine-2-phosphonic acid)

Compound III (1,2,4-triazole-3-sulphonic acid) was inactive, however, pyridyl derivatives of this compound which consequently had two possible basic groups (the triazole ring or the nitrogen atom in the pyridine ring) were either weak excitants (compounds V and VI) or reduced both glutamate and aspartate mediated excitation (compound IV), depending upon the position of the pyridine ring. Pyridine-2-phosphonic acid (compound VII) also reduced both types of amino acid excitation.

(c) Compounds VIII - XI (uracil derivatives)

DL-Willardine (compound VIII) was a weak excitant and the structure of this uracil derivative has a close resemblance to that of glutamate, one of the oxygen atoms of the uracil moiety being the second anionic group. Derivatives of uracil produced by substitution of the 2-aminoethyl group for one of the nitrogen bound protons of the uracil molecule produced either an inactive compound (compound IX) or a compound which reduced both glutamate and aspartate mediated excitation, depending on which nitrogen atom carried the substituent. 1,3-Di-(2-aminoethyl) uracil (compound XI) also reduced excitation due to both glutamate and aspartate. Although compound XI did show some selectivity in reducing excitation due to L-glutamate (Fig. 30), it was not a consistent observation and furthermore this compound tended to reduce the height of extracellularly recorded action potentials, a procaine-like action (Curtis and Phillis, 1960). The type of experiment illustrated in Fig. 30 was used to determine whether a potential glutamate antagonist would differentiate between excitation of a neurone by L-glutamate and excitation by L-aspartate. The interneurone in Fig.30 was alternately excited by electrophoretic pulses of L-glutamate (35 nA) and L-aspartate (40 nA); during the continuous administration of compound XI the excitation due to both of these amino acid excitants was reduced, but that due to L-aspartate was affected to a lesser degree.

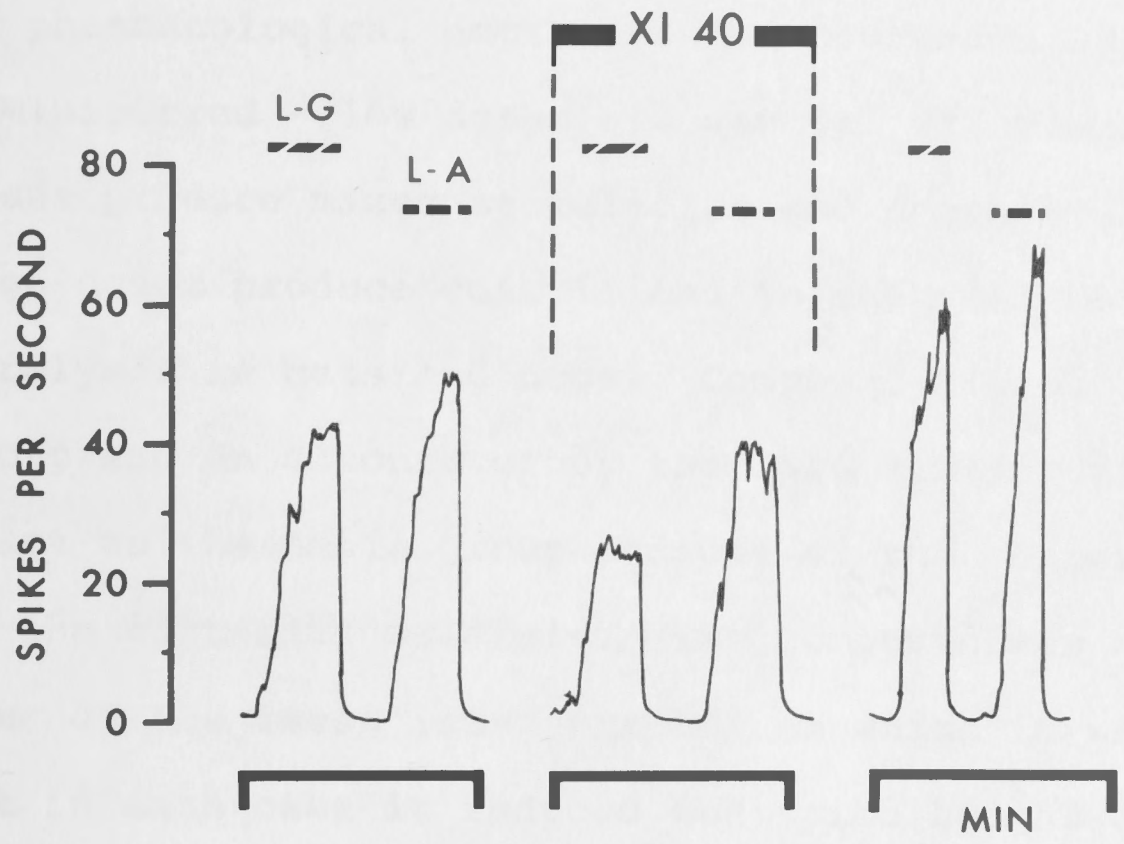


Fig. 30:

Effect of compound XI (1,3-di-(2-aminoethyl)-uracil) on the amino acid excitation of a spinal interneurone. The neurone was excited by alternate electrophoretic pulses of L-glutamate (L-G, 35 nA) and L-aspartate (L-A, 40 nA), indicated by the respective broken horizontal lines. Compound XI (40 nA) was administered for the period indicated by the horizontal bar and broken vertical lines.

(d) Compound XII

Compound XII is one of a series of diphenylamino propanols synthesised and studied by Keasling and Moffett (Keasling and Moffett, 1971). These compounds are reported to have an unusual dose-related spectrum of pharmacological activity when systemically administered - low doses are central stimulants, moderate doses produce mixed stimulation and depression, whilst high doses produce convulsions in rats and mice and paralysis in cats and dogs. Compound XII has a basic group and an anionic group that are similar distances apart to the basic group and one of the carboxyl groups in the glutamate molecule. The compound was active on four of the seven interneurones on which it was tested, but in each case it reduced the spike height as well as antagonising the excitatory action of glutamate.

(e) Compounds XIII - XVIII

In view of the reported activity of L-glutamate diethyl ester as a glutamate antagonist (Haldeman, Huffman, Marshall and McLennan, 1972) it is likely that other esters of the excitatory amino acids may be glutamate or aspartate antagonists. L-Glutamic acid di-tert-butylester (compound XIII), however, antagonised the excitatory actions of both amino acids and acetyl-choline and also reduced the amplitude of extracellularly recorded action potentials. Diethyl-DL-aspartic acid (compound XVI) was inactive when tested on eight neurones.

It is likely that esters derived from amino acids that are stronger excitants than L-glutamic acid would have a higher affinity for the receptor(s) than L-glutamic acid diethylester. N-Methyl-DL-aspartic acid is about 3-6 times as potent as L-aspartic acid (Curtis and Watkins, 1963), however, N-methyl-DL-aspartic acid diethylester (compound XVII) and N-methyl-DL-aspartic acid di-n-propylester (compound XVIII) were non-specific depressants of spinal neurones. γ-Fluoro-glutamate is a more potent excitant of spinal neurones than L-glutamate (Curtis, Duggan, Felix, Johnston, Tebēcis and Watkins, 1972), however 4-fluoroglutamate diethylester (compound XIV) was a non-specific depressant, reducing excitation by amino acids and acetylcholine. 4-Fluoroglutamic acid-1-amide (compound XV) was also inactive. The lack of specific glutamate or aspartate antagonism displayed by the esters that were tested suggests that L-glutamate diethyl ester may be unique among the esters of excitatory amino acid ester, if indeed it is a specific glutamate antagonist. Some esters of kainic acid have yet to be investigated.

(f) Compound XIX (Ambenoxan)

Ambenoxan (compound XIX) is a 2-substituted aminomethyl-1,4-benzodioxane the central actions of which are critically dependent upon the dose level (Meldrum and Bhargava, 1968; Green, Shapiro and Wilson, 1969). In the rabbit, rapid intravenous injections of low doses of ambenoxan (less than 10 mg/kg) produce

sedation and loss of muscle tone, whilst high doses produce cortical seizure followed by sedation and loss of muscle tone. The straight alkoxy side chain was apparently essential for the muscle relaxant activity and increasing the length of the chain increased the convulsant activity. Ambenoxan, which has four possible electronegative centres (the oxygen atoms) and one electropositive centre (the nitrogen atom) was a non-specific depressant reducing the excitation of inter-neurones to L-aspartate and L-glutamate, and reducing the excitation of Renshaw cells by the amino acids, acetylcholine and by ventral root stimulation. Furthermore, when electrophoretically administered, this compound consistently reduced the amplitude of extracellularly recorded action potentials.

(g) β-(p-Chlorophenyl)-GABA (Lioresal) and β-(m-trifluoromethylphenyl)-GABA

β-(p-chlorophenyl)-GABA has been separately investigated as a depressant of neuronal activity and the results of those investigations are reported elsewhere in this thesis (Section III). However, the compound (compound XXI) was also included in this series for testing as a potential glutamate antagonist. This compound depressed glutamate, aspartate and acetylcholine induced neuronal activity. β-(m-trifluoromethylphenyl)-GABA)compound XX) similarly depressed glutamate and aspartate induced neuronal activity.

(h) Compounds XXIII (Methaqualone) and XXII (Metacaine).

Methaqualone (compound XXIII) has been described as a potent hypnotic and anticonvulsant and in addition it reduces spinal polysnaptic reflexes (Swift, Dickens and Becker, 1960). A mechanism of action of this compound at the cellular level has apparently not been reported in the literature, and in these tests methaqualone was inactive when administered to individual neurones. Similarly, a mechanism of action has not yet been reported for Metacaine (compound XXII; Tricaine) which has been effectively used as an anaesthetic for fish and some other cold-blooded animals (Ball and Cowen, 1959; Stefanova, Puchta and Romanovsky, 1962). Low concentrations of metacaine are required to completely immobilise fish, and the mode of action of metacaine could be partly explained by interruption of neuromuscular transmission, possibly by a presynaptic action (Maeno, 1966). Its effects, however, on central neurones have not been reported. When tested on four Renshaw cells, metacaine consistently reduced the excitatory action of both L-glutamate and acetylcholine and significantly reduced the height of the action potential. The compound was thus a non-specific neuronal depressant, similar to procaine (Curtis and Phillis, 1960).

(i) Compound XXIV (nuciferine)

Nuciferine (compound XXIV) is structurally related to 2-methoxy aporphine which antagonises the excitant action of L-glutamate on central neurones with minimal effect on that of acetylcholine (Curtis, Duggan, Felix, Johnston, Tebēcis and Watkins, 1972). The use of 2-methoxyaporphine, however, is technically difficult due to its low aqueous solubility and furthermore, it generally antagonises L-aspartate and L-glutamate excitation to the same extent. Hence, nuciferine, which has been shown to be a CNS depressant producing similar effects to chlorpromazine (Macko, Douglas, Weisbach and Waltz, 1972) was tested as a potential glutamate antagonist. Nuciferine was tested on four Renshaw cells and consistently reduced the excitatory activity of both L-glutamate and acetylcholine, a finding that agreed with a recent report concerning its activity on rat Renshaw cells (Duggan, Lodge, Biscoe and Headley, 1973).

(j) L-Methionine-DL-sulphoximine phosphate (MSO phosphate, compound XXV)

L-Methionine-DL-sulphoximine (MSO) has been thoroughly tested as an excitatory amino acid antagonist and it has been shown to have limited usefulness as such an antagonist because of technical difficulties associated with its low aqueous solubility (Curtis, Duggan, Felix, Johnston, Tebēcis and Watkins, 1972). MSO is a convulsant when administered systemically and inhibits brain glutamine synthetase. This inhibition

is associated with the conversion of methionine sulphoximine to methionine sulphoximine phosphate which is tightly bound to the enzyme (Rowe, Ronzio and Meister, 1969; Rao and Meister, 1972) and it seems likely that the phosphoryl moiety of MSO phosphate promotes the attachment of the compound to the enzyme (Rao and Meister, 1972). Thus MSO phosphate was tested as a potential glutamate antagonist. The action of MSO phosphate was tested on a total of ten neurones with both anionic and cationic currents. Anionic currents were either inactive or reduced the height of action potentials, whilst the results with cationic currents were inconsistent, some times producing excitation and in other tests producing non-specific depression. It thus appeared that MSO phosphate was even less useful than MSO as a glutamate or excitant amino acid antagonist.

(2) 1-Hydroxy-3-aminopyrrolidone-2 (HAP)

The effects of increasing concentrations of HAP (freshly prepared, 0.2 M, pH 5-6, HCl added) on the chemical sensitivity of a Renshaw cell are illustrated in Fig. 31 and similar results were obtained with 21 other Renshaw cells (total of nine preparations). Relatively low cationic currents (10-20 nA) reversibly reduced the excitatory effects of glutamate, aspartate and DLH in a parallel manner in the absence of a change in the sensitivity to acetylcholine, whilst higher concentrations of HAP (above 20 nA) invariably reduced the excitatory action of acetylcholine on Renshaw cells. With the

Renshaw cell illustrated the concentration of HAP adequate to abolish the amino acid excitation also reduced acetylcholine excitation to less than 50% of control. Higher concentrations of HAP (above 150 nA: not illustrated) completely abolished the effects of acetylcholine. Excitation by glutamate, aspartate and DLH appeared to be equally sensitive to HAP; the seemingly greater sensitivity of the action of DLH on the cell used for Fig. 31, probably resulted from the lower frequency of excitation by this amino acid (Fig. 31A, F). Nine cells were carefully tested to determine the relative sensitivity of acetylcholine and glutamate excitation to HAP and concentrations of HAP adequate to reduce the sensitivity to glutamate by 50-70%, reduced the excitant action of acetylcholine by 15-60%. Three Renshaw cells were firing spontaneously and this firing was depressed by amounts of HAP which influenced amino acid excitation of these cells. The synaptic excitation of Renshaw cells by volleys in ventral and dorsal roots (Curtis and Ryall, 1966b) could also be reversibly reduced by HAP (6 Renshaw cells). Although the intense cholinergic excitation evoked by maximal stimulation of ventral root fibres was much less sensitive to HAP than non-cholinergic firing resulting from stimulation of peripheral afferents or dorsal root fibres, submaximal ventral root responses were only slightly less sensitive than responses of a similar magnitude evoked by dorsal root volleys (Fig. 32), but this differential sensitivity to HAP is consistent with

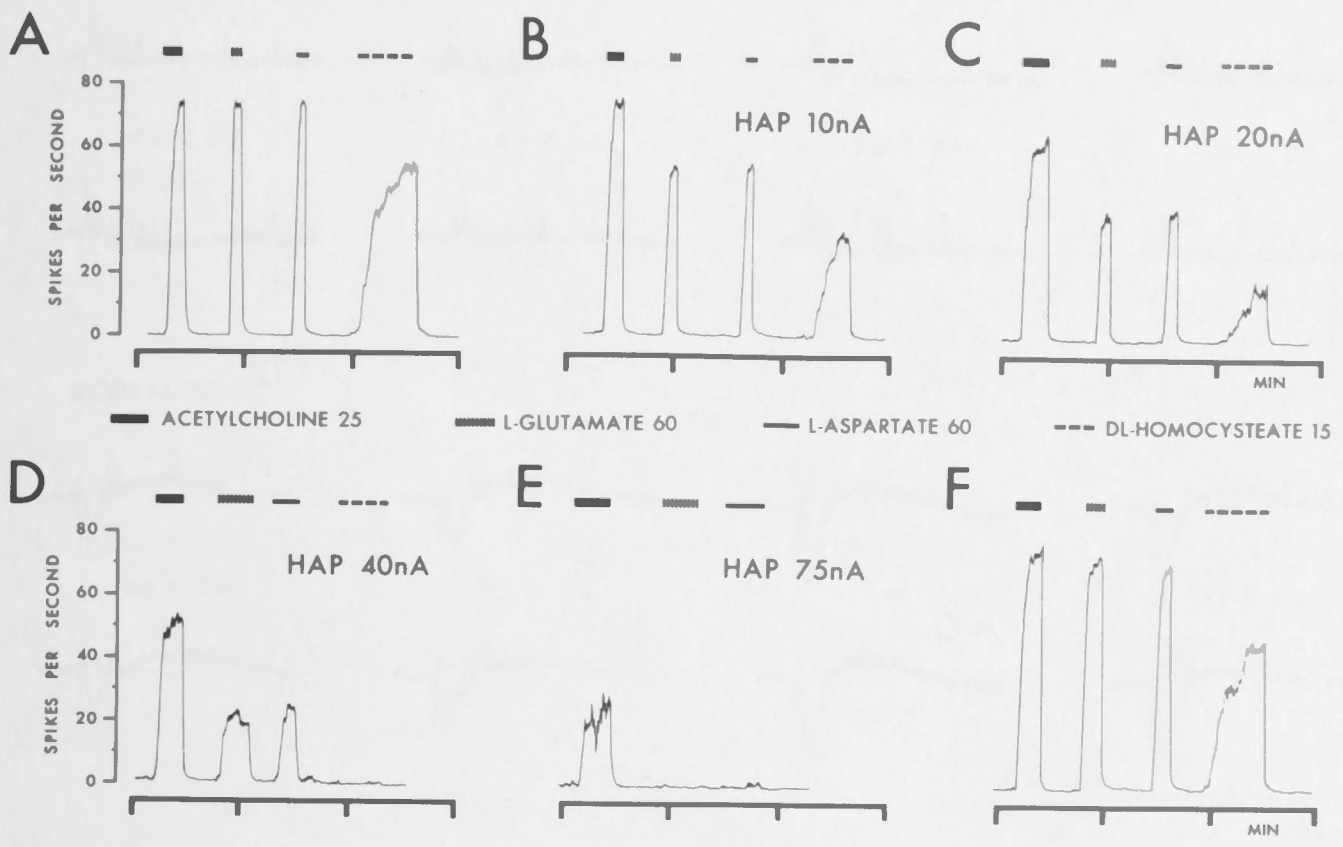


Fig. 31:

Effects of 1-hydroxy-3-aminopyrrolidone-2 (HAP) on the excitation of a Renshaw cell by electrophoretically administered acetylcholine (25 nA), L-glutamate (60 nA), L-aspartate (60 nA) and DL-homocysteate (15 nA). Times of administration are indicated by the horizontal solid, dotted and dashed lines above the records. A, control observations; B - E, HAP was administered with progressively increasing cationic currents for periods of 3-4 min. Each series of records shows the maximum effect at each dose level. F, 4 min after termination of the HAP current.

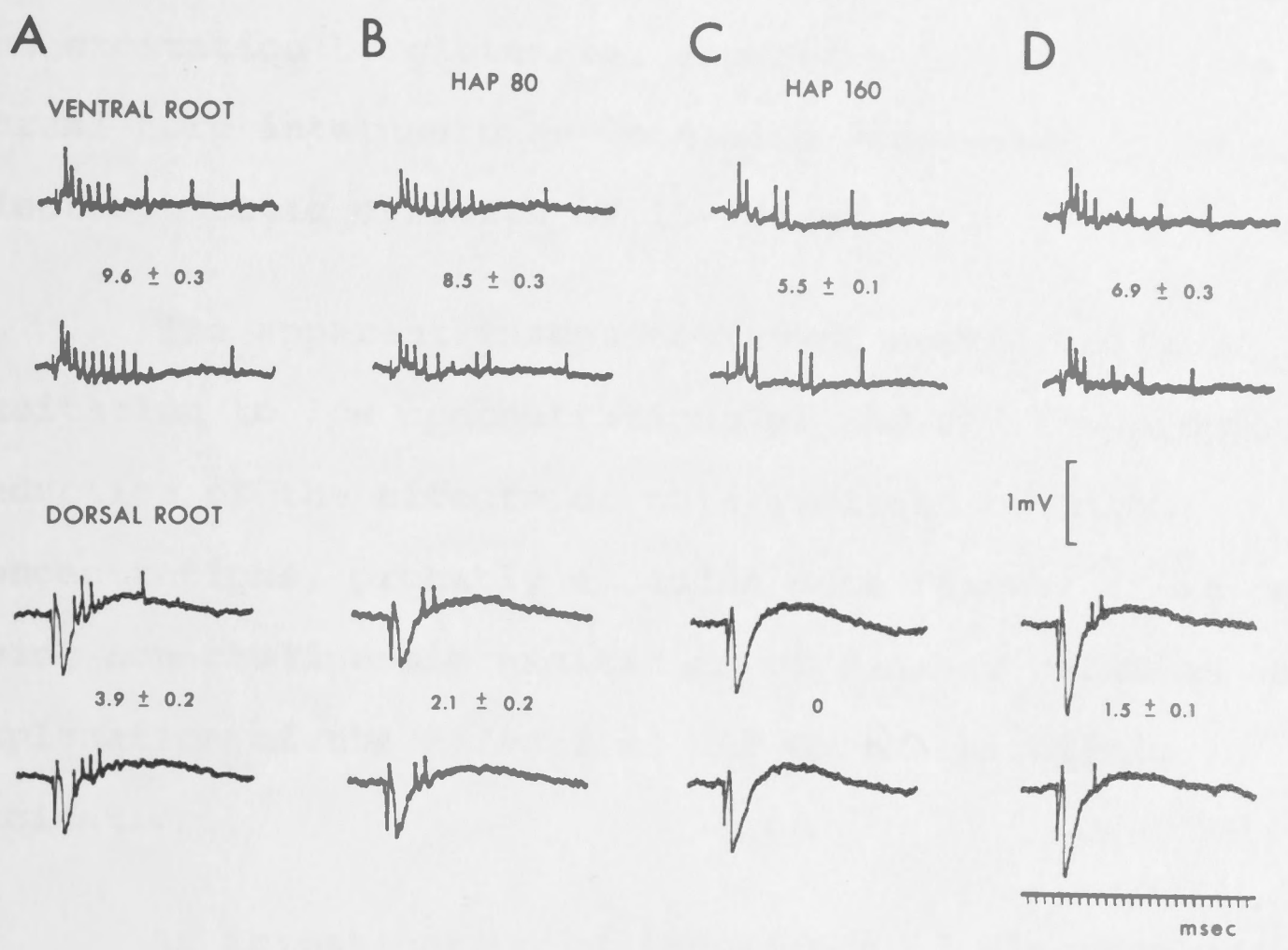


Fig. 32:

Effect of 1-hydroxy-3-aminopyrrolidone-2 (HAP) on the synaptic excitation of a Renshaw cell by volley in ventral roots (upper two rows, cholinergic excitation) and dorsal roots (lower two rows, non-cholinergic). A, control; B, during HAP 80 nA; C, during HAP 160 nA; D, incomplete recovery 4.5 min after terminating the HAP ejection and just prior to accidental displacement of the recording electrode from the vicinity of the neurone. The figures within each row give the mean number of spikes in 20 responses (± S.E.M.); the records were selected to illustrate the mean responses. The depressant effect of HAP are statistically significant ($P<0.001$ by Student's t test), apart from the ventral root responses of column B ($P\simeq0.012$). Calibrations: 1 mV, msec.

the proposition that the dorsal root excitation of Renshaw cells is non-cholinergic and possibly involves amino acids (Curtis and Ryall, 1966b). The spontaneous firing and excitation by glutamate, aspartate and DLH of five dorsal horn interneurones were also depressed by HAP using electrophoretic currents of 20-100 nA.

The apparent insensitivity of acetylcholine excitation to low concentrations of HAP and the progressive reduction of the effects of this excitant by higher concentrations, probably excludes mere removal of an underlying non-cholinergic excitation of Renshaw cells as an explanation of the effects of HAP on the nicotinic excitation.

An investigation of the effect of electrophoretically administered HAP on the sensitivity of identified neurones of the cerebral cortex (four pyramidal tract neurones: Fig. 33 illustrates one of these) and ventrobasal thalamus (eight thalamocortical relay neurones: Fig. 34 illustrates one of these) to acetylcholine and excitant amino acids yielded results that were similar to those obtained on spinal neurones. Acetylcholine excitation tended to be less sensitive to low concentrations of HAP than was amino acid excitation (except perhaps in the ventrobasal thalamus), but although the degree of selectivity was less than that required for a selective antagonist of amino acid excitation Davies and Watkins (1973) have reported that the degree of selectivity for HAP, in the feline cerebral cortex, was higher than that for either glutamate diethyl ester or methionine sulphoximine. Furthermore,

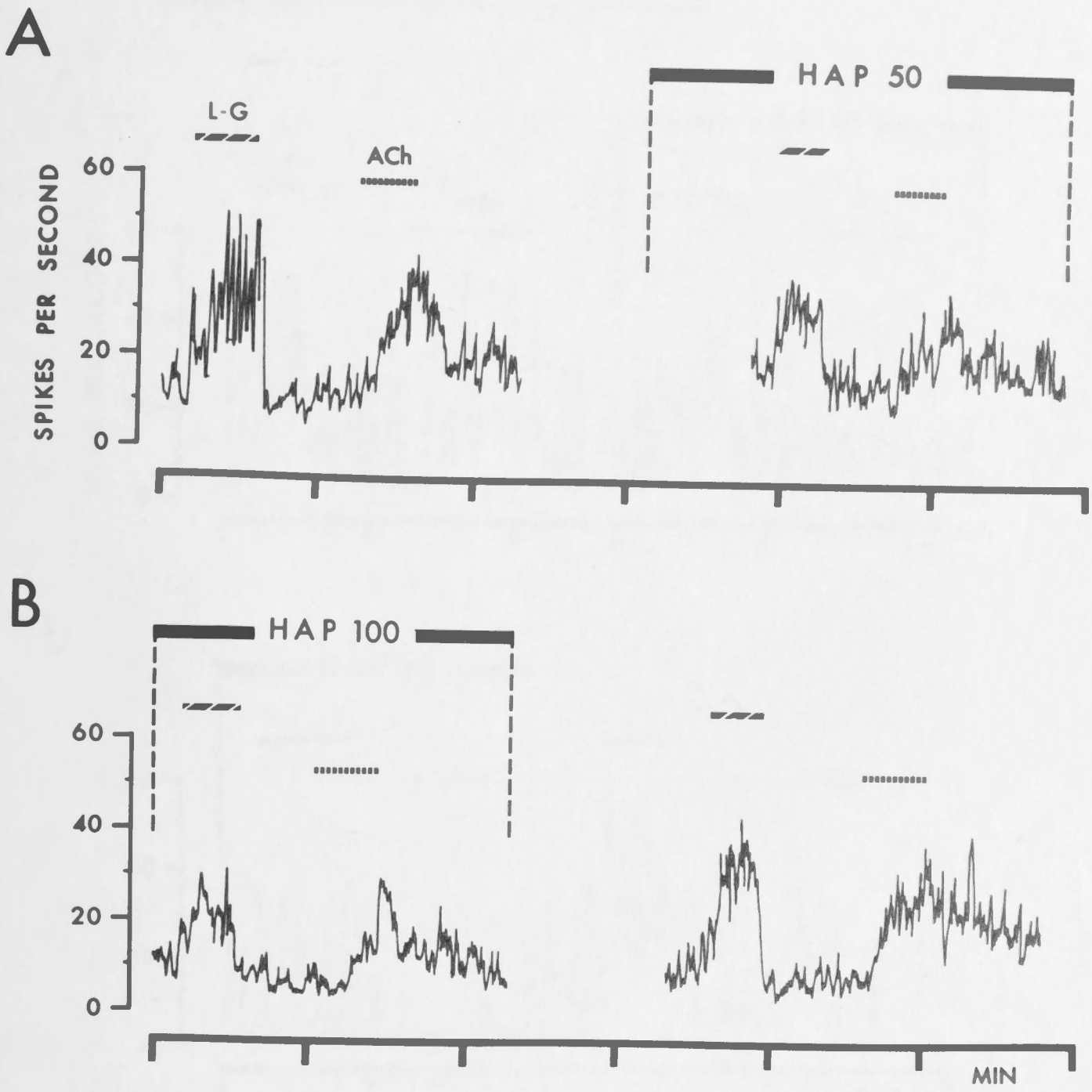


Fig. 33:

Effects of 1-hydroxy-3-aminopyrrolidone-2 (HAP) on the excitation of a pyramidal tract neurone of the post-cruciate cortex by electrophoretically administered L-glutamate (L-G, 30 nA) and acetylcholine (ACh, 100 nA). Times of administration are indicated by the horizontal solid and broken lines above the records; the times of ejection of HAP are also indicated by the vertical broken lines. A, responses before and during the administration of HAP (50 nA); B, responses during and after HAP (100 nA). 3/4 min elapsed between A and B, however, the ejection of HAP was continuous.

Fig. 34:

Effect of 1-hydroxy-3-aminopyrrolidone-2 (HAP) on the excitation of a thalamo-cortical relay neurone of the ventrobasal thalamus by electrophoretically administered L-glutamate (L-G, 50 nA) and acetylcholine (ACh, 25 nA). Times of administration are indicated by the horizontal solid and broken lines above the records and the times of ejection of HAP are further indicated by the vertical broken lines. A, responses before and during HAP (20 nA); B, responses during HAP (40 nA). A and B are successive runs, HAP being ejected for a total period of 6 min.

HAP in the spinal cord, cerebral cortex and ventrobasal thalamus equally reduced the effects of both naturally occuring excitant amino acids, glutamate and aspartate, (and that of DLH), and thus would be of no use in distinguishing these putative transmitters from each other.

On the basis of comparison of electrophoretic currents, HAP was much less effective as a neuronal depressant than either glycine or GABA on spinal neurones (Fig. 35A) and HAP has been reported to be a weaker depressant than glycine or GABA in the cuneate nucleus and weaker than GABA in the cerebral cortex (Davies and Watkins, 1973). The relative weakness of HAP as a neuronal depressant has presented difficulty in determining whether it is a bicuculline- or strychnine-sensitive depressant. However, studies on four Renshaw cells indicated that strychnine did not reduce the depressant effect of HAP and that BMC, in concentrations sufficient to block or markedly reduce the inhibitory action of GABA, had considerably less effect on that of HAP (Fig. 35B). This finding was in agreement with Davies and Watkins (1973) who found that HAP was not greatly affected by strychnine in the cuneate nucleus, or by bicuculline in the cerebral cortex and cuneate nucleus. In general glycine and GABA depress the excitation of Renshaw cells by glutamate, aspartate, DLH and acetylcholine to the same extent, thus the small degree of selectivity of HAP as an excitant amino acid antagonist and the failure of BMC and strychnine to reduce, to a significant extent, its depressant action suggests that its mechanism of action is basically different from that of glycine or GABA.

RENSHAW CELL

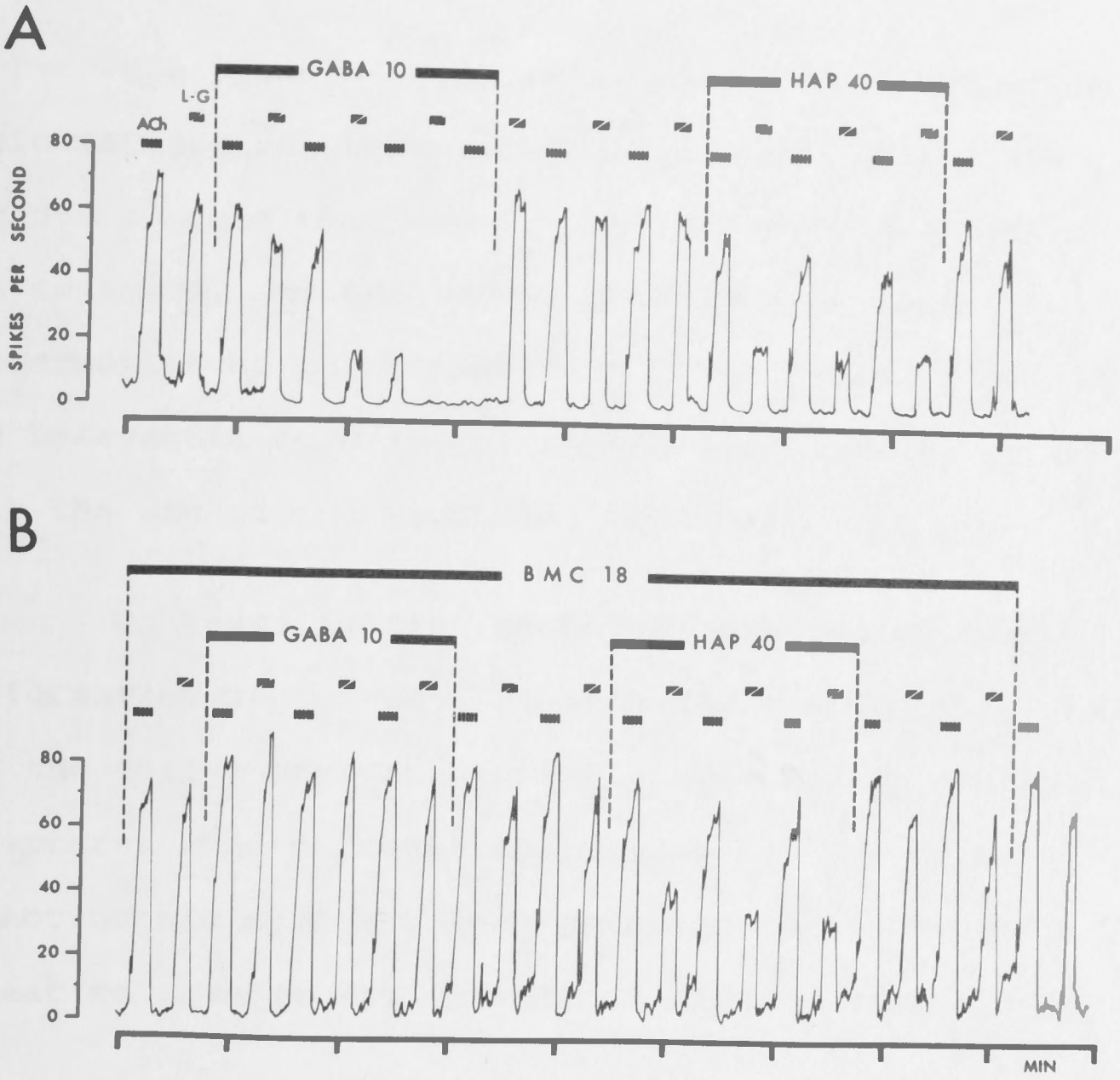


Fig. 35:

Effect of bicuculline methochloride (BMC) on the depressant action of 1-hydroxy-3-aminopyrrolidone-2 (HAP) and GABA on a Renshaw cell excited by acetylcholine (ACh) and L-glutamate (L-G). The cell was excited by alternate electrophoretic pulses of acetylcholine (0 nA = no retaining current) and L-glutamate (60 nA), the horizontal broken lines indicate the periods of ejection of excitants. The horizontal solid bar and vertical broken lines indicate the periods of ejection of GABA, HAP and BMC (10 mM in 165 mM NaCl). A, responses to ACh and L-G before, during and after GABA (10 nA) and before, during and after HAP (40 nA); B, the effect of BMC (18 nA) on the depressant actions of GABA (10 nA) and HAP (40 nA).

SUMMARY

1. The present state of knowledge concerning the conformations of gamma-aminobutyric acid (GABA) and L-glutamic acid that are active in biological systems was reviewed, and the use of analogues of restricted conformation to further analyse GABA, L-glutamic acid and L-aspartic acid receptors was discussed in parallel with the use of the microelectrophoretic method.

2. An investigation of GABA analogues of restricted conformation suggested that extended conformers of GABA are the active ones at bicuculline-sensitive postsynaptic receptors. The neuronal depressant actions of cis-4-aminocrotonic acid and β-(p-chlorophenyl)-GABA do not appear to involve either GABA or glycine receptors.

3. Of the several bicuculline analogues tested for selective GABA antagonism, bicuculline methochloride (BMC) was found to be more suitable and more potent than bicuculline as a microelectrophoretically administered GABA antagonist. The central effects of bicuculline and BMC could not be explained on the basis of a weak anti-cholinesterase effect, measured *in vitro*, and it seems more reasonable to consider that these effects are due to antagonism of GABA-like amino acids.

4. A possible explanation of the convulsant actions of tutin, shikimin, coriamyrtin and penicillin was found to be antagonism of GABA-like amino acids. Whilst these

convulsants may antagonise glycine-like amino acids when in relatively high concentrations, d-tubocurarine was non-selective and antagonised glycine and GABA at all 'dose' levels.

5. Kainic acid was found to be the most potent excitant amino acid presently known, and there is a unique juxtaposition of the equivalent ionisable groups in L-glutamic acid and the following analogues of restricted conformation: kainic, 'cycloglutamic' and ibotenic acids.

6. With sensitivity to N-methyl-D-aspartate as a basis of comparison, spinal interneurones were relatively more sensitive to kainate than were Renshaw cells, a finding which provides additional support for the proposition that glutamate could be the excitatory transmitter at primary afferent synapses in the spinal cord.

7. A number of possible antagonists of the excitatory action of L-glutamic acid were tested, including the reported antagonist 1-hydroxy-3-aminopyrrolidone-2(HAP), but none was found to be sufficiently selective to be a useful glutamate antagonist.

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